IDENTIFICATION OF BOTH STRUCTURAL AND FUNCTIONAL DOMAINS OF THE SENDAI VIRUS NUCLEOCAPSID PROTEIN REQUIRED FOR VIRAL RNA REPLICATION

By

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KEY TO SYMBOLS

aa	amino acid
ATP	adenosine 5'-triphosphate
bp	base pair
C-	carboxy
°C	degrees centigrade
CCR	central conserved region
cfu	colony forming units
Ci	curie
cm	centimeter
cpm	counts per minute
cs	cold sensitive
CTP	cytidine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DI	defective interfering particle
DNA	deoxyribonucleic acid
ds	double stranded
GTP	guanosine 5'-triphosphate
h	hour
I.U.	international unit
kb	kilobase
kDa	kilodalton

µg or mcg	microgram
µCi	microcurie
µl	microliter
µm	micrometer
µM	micromolar
mCi	millicurie
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
min	minute
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
(-)	minus sense
ng	nanogram
nt	nucleotide
OD	optical density
^{32}P	phosphorus 32
pfu	plaque-forming units
(+)	plus sense
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
^{35}S	sulfur 35
ss	single stranded
TCA	trichloroacetic acid

ts	temperature sensitive
TTP	thymidine 5'-triphosphate
tRNA	transfer ribonucleic acid
U	units
UV	ultraviolet
UTP	uridine 5'-triphosphate
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

IDENTIFICATION OF BOTH STRUCTURAL AND FUNCTIONAL DOMAINS OF THE SENDAI VIRUS NUCLEOCAPSID PROTEIN REQUIRED FOR VIRAL RNA REPLICATION

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The nucleocapsid protein (NP) of Sendai virus encapsidates the genome RNA during viral replication forming a helical nucleocapsid (Nuc), which is the template for RNA synthesis by the viral polymerase. Consequently, the NP protein has both structural and functional roles and it is an essential component of the NP-P (P, phosphoprotein), NP-NP, Nuc-P/L (L, large protein), and NP-RNA complexes required during viral RNA replication. To identify domains in the NP protein, six mutants were constructed using charge-to-alanine mutagenesis in a highly charged region from amino acids (aa) 107 to 129. All the mutants were active in the first round of RNA replication; however, the product nucleocapsids formed with three mutants, NP114, NP121, and NP126, did not serve as

templates for amplification. The template defect was not due to a lack of protein-protein interactions or polymerase binding to the nucleocapsid.

Further alanine substitutions at conserved hydrophobic residues in the mutants NP362 and NP364 disrupted the NP-NP interaction, suggesting these aa are required for NP-NP complex formation. Another charge-to-alanine mutant, NP370, was completely inactive in replication *in vitro*. Since this mutant formed all the essential protein-protein complexes, we propose that the mutant NP370 is defective in the NP-RNA interaction.

Sedimentation analysis of fusions between the maltose-binding protein (MBP) and portions of the NP protein (MBP.NP) identified the central conserved region (CCR, aa 258-357) (MBP.NP1) as containing a NP-NP binding domain. The MBP.NP1 fusion protein, but not the MBP alone, was also shown to inhibit viral RNA replication. Site-directed mutagenesis of the CCR in the full-length NP protein showed that the mutants NP260-1, NP324-1, and NP324-5, like NP362 and NP364, were defective in the NP-NP interaction. Mutants NP299-5 and NP313-2, like NP370, formed protein-protein complexes, but were inactive in replication, suggesting the NP-RNA interaction was disrupted. These results show that although the NP-NP and NP-RNA domains may overlap they can be separated genetically, suggesting the residues required for each interaction are discrete.

CHAPTER 1
INTRODUCTION AND BACKGROUND

Viral Taxonomy

Sendai virus is a member of the family *Paramyxoviridae*. The *Paramyxoviridae*, *Rhabdoviridae*, and *Filoviridae* families contain viruses with nonsegmented single-stranded (ss) negative sense RNA genomes. One of the characteristic features of these viruses is that the genome is tightly associated with the nucleocapsid protein, forming a helical nucleocapsid, which is the template for RNA synthesis (for a review see Kingsbury, 1991). The ss negative sense (-) RNA viruses are similar in terms of genomic size, organization, and content (Pringle, 1991). The family *Paramyxoviridae* includes three genera, the *Paramyxovirus*, *Morbillivirus*, and *Pneumovirus*. The paramyxoviruses and morbilliviruses are closely related in both genomic content and structure, as well as viral morphology, as compared to the pneumoviruses (Pringle, 1991). The genetic content of the vesicular stomatitis viruses of the *Rhabdoviridae* family is also quite similar to the paramyxoviruses (Galinski and Wechsler, 1991). Members of the *Paramyxovirus* genus include the human, bovine, and avian parainfluenza viruses (PIV), Newcastle disease virus (NDV), mumps virus (MuV), simian virus 5 and 41 (SV5 and SV41), and Sendai virus (SV). The *Morbillivirus* genus

contains the measles (MV), canine distemper (CDV), rinderpest, and peste des petits ruminants viruses, while the respiratory syncytial viruses (RSV; human and bovine) and pneumonia virus of mice are members of the *Pneumovirus* genus.

Several of these viruses including the human parainfluenza viruses, mumps virus, measles virus, and respiratory syncytial viruses are communicable human pathogens that often result in acute childhood illnesses. Even with the availability of vaccines for some of the diseases, such as measles and mumps, these viruses continue to be associated with significant morbidity and mortality worldwide (Black, 1991; Huber et al., 1991; Collins et al., 1993; Fooks et al., 1993). Subacute sclerosing panencephalitis (SSPE), which is almost always fatal, has unequivocally been shown to be due to persistence of a measles virus infection (Billeter and Cattaneo, 1991; Huber et al., 1991). In addition, several chronic human diseases, including multiple sclerosis, autoimmune chronic active hepatitis, and Paget's disease have been tentatively associated with the presence of persistent infections established by these viruses (Billeter and Cattaneo, 1991; Randall and Russell, 1991). The significance of these diseases is sufficient to justify continued research on these viruses, but when combined with the limited understanding of how these viruses regulate RNA synthesis, it is evident they deserve our investigation.

Genomic Structure

Sendai virus is a murine pathogen that produces an illness in mice (pneumonia) similar to that seen with human parainfluenza I (in humans) and it is used as a model paramyxovirus (Parker and Richter, 1986). The complete nucleotide (nt) sequence (15,384 nt) of Sendai virus has been determined and the genes for the six major proteins have been mapped in the following order: 3' NP-P/C/V-M-F_o-HN-L 5' (Galinski and Wechsler, 1991; Moyer and Horikami, 1991). The P gene is unique in that it also codes for several nonstructural proteins C, C', Y_1, Y_2, and X from overlapping reading frames (Curran et al., 1991a; Lamb and Paterson, 1991), as well as the V and W proteins from polymerase editing (Vidal et al., 1990b; Horikami and Moyer, 1991; Lamb and Paterson, 1991). The viral genome contains extracistronic leader sequences (54 nt) at the 3' and 5' ends. In VSV these sequences have been shown to contain the promoter and encapsidation signals (Blumberg et al., 1983; Kolakofsky et al., 1991; Moyer et al., 1991, Smallwood and Moyer, 1993).

Sequence analysis of all the known paramyxoviruses has shown the first 11 nucleotides of the genome (-) and antigenome (+) 3' ends are highly conserved, suggesting they may act as similar recognition sequences (Blumberg et al., 1991). Conserved sequences are also found at each of the gene junctions and in Sendai virus the consensus sequences are 3'-gene-UNAUUCUUUUU GAA UCCCANUUUC-gene-5' (Galinski and

Wechsler, 1991; Moyer and Horikami, 1991). The first 11 nucleotides following a gene are believed to contain the putative polyadenylation signal. It is followed by a nontranscribed intergenic sequence of 3 nt, which is followed by the start signal for the next.

The Virion and the Viral Lifecycle

The virion of the paramyxoviruses is composed of the nucleocapsid, which is surrounded by the viral envelope (for a review see Kingsbury, 1991). The viral glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F_1 and F_2) proteins are inserted into the host-derived virion envelope and are the proteins that mediate attachment and fusion of the virus to the cellular receptor during infection (Pringle, 1991). The matrix protein (M) is associated with the inner surface of the viral envelope and it is believed to function as a bridge between the nucleocapsid core and the plasma membrane during virus budding (Galinski and Wechsler, 1991). Support for this theory is based on the possible interaction of the basic matrix protein (+14 to +17 at neutral pH) with the acidic NP protein (Morgan et al., 1984; Galinski and Wechsler, 1991).

Approximately, 2600 molecules of the nucleocapsid (NP, 524 aa) protein are tightly associated with the genomic RNA, resulting in the formation of a nuclease resistant helical nucleocapsid (RNA-NP) (Heggeness et al., 1980; Galinski and Wechsler, 1991). The nucleocapsid is roughly 15 nm in diameter and 1 µm in length (Galinski and Wechsler, 1991).

The viral RNA-dependent RNA polymerase is composed of two subunits, the phosphoprotein (P, 568 aa) and the large protein (L, 2228 aa) (Hamaguchi et al., 1983; Gotoh et al., 1989; Galinski and Wechsler, 1991). Approximately 300 molecules of the P protein and 30 molecules of the L protein are loosely associated with the nucleocapsid (P/L-Nuc) (Galinski and Wechsler, 1991). Electron microscopy and biochemical data suggest that the L protein is associated with the nucleocapsid through its interaction with the P protein (Portner and Murti, 1986; Portner et al., 1988; Morgan, 1991; Horikami and Moyer, 1995). The presence of the P-L complex on the nucleocapsid could increase the rapidity with which viral RNA synthesis is initiated following infection.

Following attachment and fusion of the virus with the cell membrane the nucleocapsid is released directly into the cytoplasm, unlike the togaviruses, orthoviruses, rhabdoviruses, and retroviruses, which enter through endocytotic vesicles (Galinski and Wechsler, 1991). The viral polymerase initiates transcription and the viral mRNAs are translated by the cellular translational machinery. Transcription and translation are followed by the replication of the negative sense (-) viral genome producing a positive strand (+) intermediate (antigenome), which then serves as the template for (-) strand replication. The genome and antigenome are each encapsidated by the NP protein, forming nuclease-resistant nucleocapsids (Kolakofsky et al., 1991).

Both transcription and replication are discussed in more detail below. The association of the nucleocapsid with the matrix protein and the association of the matrix protein with the plasma membrane is not clearly understood, but it is believed the matrix protein positions the nucleocapsid with the HN and F containing plasma membrane (Galinski and Wechsler, 1991). The final step of virion maturation is accomplished by budding through the plasma membrane.

Both the NP and P proteins are the primary phosphorylated proteins in the virion (Hsu and Kingsbury, 1982). Since the NP protein will be discussed in depth below, it will be not be included in this section. The majority of phosphate residues in the P protein are located in a N-terminal 18 kDa fragment (Hsu and Kingsbury, 1982). While the role of phosphorylation of the P protein is not known, the N-terminal 18 kDa fragment does not appear to be required for transcription (Kolakofsky et al., 1991). Recently, Gao and Lenard (1995) reported that for the VSV P protein, phosphorylation at the S60 and T62 residues was linked to oligomerization of the VSV P protein as well as transcriptional activation. The data indicated that phosphorylation was required for VSV P protein oligomerization and that the multimeric VSV P protein was the transcriptionally active form.

The L protein is presumed to contain all the enzymatic activities of the viral polymerase, including mRNA capping, methylation, and polyadenylation, analogous to the VSV L

protein in which these enzymatic activities have been identified (Hammond and Lesnaw, 1987; Hercyk et al., 1988; Moyer and Horikami, 1991; Hunt and Hutchinson, 1993). The identification of six conserved regions in the L proteins of the paramyxoviruses and rhabdoviruses has led to the suggestion that they represent functional domains (Poch et al., 1990; Sidhu et al., 1993; Horikami and Moyer, 1995).

Deletion Mutants of the Paramyxoviruses

Defective interfering particles (DI) of the paramyxoviruses are shorter versions of the genome that require the presence of nondefective homologous helper virus to replicate (Kolakofsky, 1976; Re, 1991; Calain et al., 1992; Engelhorn et al., 1993). Analogous to wt virus, both the plus and minus sense DI RNAs are encapsidated by the NP protein provided by the helper virus and share the ribonuclease-resistant property of the nondefective nucleocapsid (Kolakofsky, 1976; Re, 1991). DI particles are produced spontaneously during infection and can be amplified by repeated high multiplicity passage in animal cells or in embryonated eggs. The smaller size and conservation of the viral RNA sequences required for replication lead to their selective replication over the helper virus (Kolakofsky, 1976; Re, 1991; Calain et al., 1992).

Two types of DI genomes have been identified and both can be isolated from Sendai virus infections (Kolakofsky, 1976; Hsu et al., 1985; Re, 1991). Internal deletion DI genomes preserve the genomic 3' and 5' ends, but the coding

sequence is extensively deleted (Hsu et al., 1985; Re, 1991). The internal deletion DI genomes contain the signals for both transcription and replication and have been shown to produce authentic mRNAs (Engelhorn et al., 1993). Another form of a DI genome is the copy-back, which contains the C-terminal portion of the L gene including the polyadenylation signal, the intergenic trinucleotide, and the 54 base leader sequence of the 5' genomic end (Re, 1991). From 50-210 nt of the 3' end is complementary to the 5' end; therefore, it is identical to the 3' end of the antigenome. This sequence complementarity is responsible for the characteristic panhandle structures observed when copy-back DI RNAs are analyzed by electron microscopy (Kolakofsky, 1976; Leppert et al., 1977). Because the copy-back DI genomes lack the regulatory transcriptional sequences found at the wt genomic 3' end they do not express functional mRNAs (Engelhorn et al., 1993). The generation of the DI genomes can be explained by the polymerase dissociating from template with the nascent RNA and either reinitiating downstream of the dissociation point (internal deletions) or reinitiating on the nascent strand (copy-back) (Re, 1991).

The internal deletion and copy-back DI particles have been used to study the regulatory *cis*-acting sequences at the termini. The copyback DI nucleocapsids have been shown to inhibit the replication of internal deletion DI particles (Re, 1991). This is not surprising since the copy-back DI particles are templates for replication only. Because the

copyback 3' end promoter is identical to the antigenome 3' end, it may be preferred because it is the promoter for the production of minus (genomic) strands. The DI particle used in the experiments described here is the copy-back type (DI-H). The DI-H genome is 1410 nt in length with 110 nt of terminal complementarity (Leppert et al., 1977, Calain et al., 1992).

RNA Synthesis

Transcription initiates at the exact 3' end of the genome producing a 54 nt leader RNA that is neither capped nor polyadenylated and six or more (depending on the number of mRNAs generated from the P gene) monocistronic, capped, and polyadenylated mRNAs (Kolakofsky et al., 1991; Horikami and Moyer, 1995). The viral polymerase is thought to terminate transcription after the leader RNA has been synthesized, skip three nucleotides, and reinitiate transcription at the NP gene. A termination signal following the leader has not been identified, but some sequence similarity does exist between the end of the leader sequence and the intergenic sequences at the gene junctions, which may serve as a termination signal (Galinski and Wechsler, 1991). Starting with transcription of the NP gene the polymerase subsequently recognizes the consensus termination and initiation sequences at each gene junction (Kolakofsky et al., 1991).

Occasionally, polycistronic transcripts containing the three base intergenic sequence but lacking polyadenosine have

been detected. Although these readthrough products can happen at any one of the gene junctions, the leader-NP junction readthroughs have been well studied (Perrault et al., 1983, Kolakofsky et al., 1991). Both the Z strain of Sendai virus and a VSV *PolR* mutant produce a much higher proportion of leader-NP transcripts than the H strain of Sendai virus or wt VSV, respectively, and these mutations have been mapped to the NP component of the nucleocapsid template (Curran et al., 1993). The lack of polyadenosine in the readthrough transcripts suggests that polyadenylation and termination of transcription are coordinately regulated (Galinski and Wechsler, 1991).

Transcription of the mRNAs is polar, such that the NP mRNA is the most abundant message and the L mRNA is the least abundant (Kolakofsky et al., 1991). The observed attenuation in transcription is probably due to some release of the viral polymerase after termination at each gene junction. Transcription, unlike replication does not require de novo protein synthesis as demonstrated by the lack of transcriptional inhibition in the presence cycloheximide (Carlsen et al., 1985; Collins, 1991). The current model for the regulation of transcription and replication is based on the availability of the NP protein (Leppart et al., 1977; Kolakofsky et al., 1991).

It is thought that the viral polymerase is responsive to the transcription initiation and termination signals until there is sufficent NP protein available to encapsidate the

nascent RNA genome. Once the critical concentration of NP is present, encapsidation of the nascent RNA is initiated and continues, concurrent with RNA synthesis. It has been proposed that encapsidation prevents the viral polymerase from responding to the start and stop signals at each gene junction (Baker and Moyer, 1988; Kolakofsky et al., 1991). The first replicative product is the (+) strand complement (antigenome) of the viral genome and the antigenome serves as the template for the production of the progeny (-) strand genomes (Kolakofsky et al., 1991). Both the genome and antigenome are encapsidated by the NP protein and resistant to ribonuclease. Once initiated, encapsidation of the nascent RNA appears to be a highly cooperative process, because even when a low concentration of the VSV N protein was incubated with the VSV leader RNA (47 nt) for short periods, only the full-length encapsidated leader was detected (Blumberg et al., 1983).

Replication can be thought to occur as follows: First is initiation of RNA synthesis and encapsidation, which occurs at the 3' end by the viral polymerase and the NP protein, respectively; second is elongation and concurrent encapsidation of the nascent RNA; and the final step is termination at the precise 5' end (Moyer and Horikami, 1991). It is not known whether the NP protein recognizes a specific sequence or structure for initiation of encapsidation, nor is it clear if the P protein is required for recognition. As discussed below, the P protein forms an NP-P complex that is

required for *in vitro* replication, and the data suggest that the NP-P complex, like the VSV N-NS complex, is the substrate for encapsidation (Peluso and Moyer, 1988; Horikami et al., 1992).

One of the least understood aspects of RNA synthesis is how the viral polymerase copies the genome in the presence of the NP protein on the template. Minimally, two models can be proposed. One is that the template "breathes" during RNA synthesis resulting in the local displacement of the NP protein (Hudson et al., 1986; Curran et al., 1993). Another possibility is that the bases are exposed in the nucleocapsid and only the phosphate backbone of the RNA is protected by the NP protein.

Initially, viral replication of the ss negative-sense RNA viruses was measured either *in vivo* or *in vitro* utilizing intracellular templates produced by wt virus infections. At this time is was not possibe to produce infectious virus from a recombinant clone. Replication was assayed *in vivo* by radiolabeling wt SV infected or wt SV plus DI-H coinfected cells (Carlsen et al., 1985). *In vitro* replication was measured by preparing cytoplasmic cell extracts from wt SV infected or wt SV plus DI-H coinfected cells and incubating the extracts with radioactive uridine to detect newly synthesized RNA. These replication assays showed that coinfection with SV DI-H, as compared to SV infection alone, produced full-length SV genome RNA (50S) at a higher frequency and full-length SV DI-H RNA (14S). Purified

detergent disrupted SV DI-H (dd DI-H) was also shown to be replicated *in vitro* by incubation with the soluble protein fraction from SV infected cells (Carlsen et al., 1985). Subsequently, purified soluble NP protein alone was shown to support replication of intracellular nucleocapsids (Baker and Moyer, 1988).

An *in vitro* replication assay that measures initiation, elongation, and termination was subsequently designed using a transient mammalian expression system and SV genes cloned downstream of the phage T7 promoter (Peluso and Moyer, 1983; Pattnaik and Wertz, 1990; Curran et al., 1991a; Horikami et al., 1992). The plasmids containing the SV genes are expressed in cells by a recombinant vaccinia virus that expresses the phage T7 polymerase (VVT7) (Fuerst et al., 1986). Using this cell-free system, it was shown that cytoplasmic cell extracts containing the Sendai virus NP, P, and L proteins could replicate purified dd DI-H virus, polymerase-free DI-H templates (RNA-NP), and intracellular nucleocapsids *in vitro*. DI-H RNA replication was dependent on the coexpression and complex formation of the NP and P (encapsidation substrate) proteins and the P and L (polymerase) proteins. Similarly, an analogous NP-P complex (N-NS) was shown to support VSV RNA replication in vitro (Peluso and Moyer, 1988; Howard and Wertz, 1989).

The differences in the requirement for the P protein during replication of intracellular nucleocapsids when the proteins are expressed from plasmids, versus biochemically

purified NP protein, is likely due to the amount of soluble NP available in the purified NP preparation (Horikami et al., 1992). Expression of the plasmid-encoded NP results in the formation of primarily large oligomeric complexes as shown by sedimentation assays and little, if any, soluble monomeric protein is observed (Horikami et al., 1992; Buchholz et al., 1993). A cosedimentation assay showed that the coexpression of the P protein with the NP protein inhibited the formation of the large oligomeric NP complexes and maintained the NP protein in a soluble form (Horikami et al., 1992; Curran et al., 1995). Similarly, coexpression of both the MV P or VSV NS proteins with their homologous N protein resulted in an N-P or N-NS complex, respectively. These complex formations also prevented oligomerization of the MV and VSV N proteins (Howard and Wertz, 1989; Huber et al., 1991; Gombart et al., 1993; Chandrika et al., 1995). It has also been shown that in SV infected cells the NP protein enters nucleocapsids through a soluble protein pool (Kingsbury et al., 1978). Although the substrate for encapsidation in the SV infected cell is not currently known these data strongly suggests it is the NP-P complex.

The *in vitro* replication assay uses an excess of input templates and it is believed to measure just one round of synthesis and encapsidation of the nascent RNA (Curran et al., 1993). An attractive feature of this expression system is that other plasmids (containing the gene of interest) can be added to, or substituted for, the wt NP, P, and L plasmids

to study the effect(s) on biological function. For example, the measles virus N protein has been shown to replicate SV DI-H, but at a reduced level (30%) compared to NP (Chandrika et al., 1995). This activity was not dependent on the coexpression of SV P and MV N since MV N does not form a complex with SV P.

The more recent reports of generating nucleocapsids using recombinant DNA technology is a significant achievement for the study of the negative strand viruses. For the first time, it has been possible to use reverse genetics to study replication and transcription. The first report was of RNA transcripts containing a reporter gene flanked by influenza A termini that were encapsidated *in vitro*. The newly assembled nucleocapsid could be transfected into cells and amplified using proteins provided by wt influenza helper virus (Luytjes et al., 1989). Since then many reports using minigenomes of different viruses have followed, some using a reporter gene and helper virus infection to provide the viral proteins required for replication (Collins et al., 1991, 1993; Park et al., 1991; De and Banerjee, 1993; Dimock and Collins, 1993). Others have utilized RNA expressed from cDNA and plasmid-encoded viral proteins (Ball, 1992; Calain et al., 1992; Pattnaik et al., 1992, 1995; Conzelmann and Schnell, 1994; Yu et al., 1995).

Infectious virus obtained from a cDNA clone containing the entire viral genome has only been reported for two of the ss negative sense viruses, VSV and rabies (Lawson et al.,

1995; Schnell et al., 1994). A disadvantage of this system is that the initial encapsidation event for both minigenomes and full-length genomes occurs at a very low frequency of ~1 in 10^2 to 1 in 10^7 transfected cells, respectively (Lawson et al., 1995; Schnell et al., 1994). Consequently, the detection of mutant viruses in which encapsidation has been compromised may require multiple transfections and will of necessity give a negative rescue.

To assay Sendai virus replication *in vivo*, a plasmid containing the cDNA of the (-) DI-H genome is cotransfected with the plasmids containing the genes for the NP, P, and L proteins into mammalian cells. The DI-H cDNA is transcribed by VVT7 polymerase and the (+) DI-H RNA is encapsidated by the expressed NP protein (Calain et al., 1992; Calain and Roux, 1993). The encapsidated DI-H RNA serves as the template for replication by the expressed NP, P, and L proteins *in vivo*. Formation of the correct 3' and 5' ends of the RNA has been most important to the success of these model systems (Ball, 1992; Pattnaik et al., 1992; Calain and Roux, 1993).

In the Sendai DI-H clone, precise positioning of the T7 promoter before the first nucleotide of the viral RNA and the insertion of the hepatitis delta ribozyme downstream of the coding sequence at the genomic end to cleave the RNA has led to the generation of T7 transcripts containing the correct 5' and 3' ends, respectively (Calain et al., 1992; Calain and Roux, 1993). Detection of the replicative products in cell

extracts is possible only after several rounds of amplification have occurred, and this can be used as a measure of the nucleocapsid to function as a template (Curran et al., 1993). Another important aspect in cloning the genomic cDNA is whether the (+) or (-), would be utilized. The formation of RNA-RNA hybrids between the (-) strand and the mRNAs transcribed from the plasmid-encoded viral proteins would inhibit encapsidation (Schnell et al., 1994). This could be the reason that a VSV genomic cDNA clone that directed the synthesis of a (-) strand T7 transcript did not yield infectious VSV (Lawson et al., 1995). Yet, cDNA clones of VSV and rabies that directed the synthesis of a (+) strand T7 transcript did produce infectious virus (Schnell et al., 1994; Lawson et al., 1995)

The NP Protein

The data presented above have established the importance of the Sendai virus NP protein in viral RNA synthesis. One can surmise that the NP protein is an essential component of both protein-protein and protein-RNA interactions required for viral RNA replication, including the NP-NP, NP-P, P/L-Nuc and NP-RNA interactions (Horikami et al., 1992; Buchholz et al., 1993; Curran et al., 1993). In addition to the phenotype of the VSV *polR* mutant mapping to the N protein in the nucleocapsid, the fact that unencapsidated RNA is not recognized by the viral polymerase is evidence that the NP protein has functional as well as structural roles in RNA replication (Emerson and Wagner, 1972; Buchholz et al.,

1993). The majority of the information on the structure of the NP protein has come from biochemical studies, such as epitope mapping with monoclonal antibodies, trypsin digestion of nucleocapsids, phoshorylation studies, and mutagenesis of the NP gene (Heggeness et al., 1980, 1981; Hsu and Kingsbury, 1982; Deshpande and Portner, 1984; Gill et al., 1988; Fisher, 1990; Homann et al., 1991; Buchholz et al., 1993, 1994; Ryan et al., 1993; Curran et al., 1993).

Examination of paramyxovirus and rhabdovirus nucleocapsids, including SV, SV5, and VSV by electron microscopy has revealed that the nucleocapsids could be tightly coiled (1.0 M NaCl) or loosely coiled (0.01 M sodium phosphate buffer) depending on the salt concentration (Heggeness et al., 1980, 1981). Digestion of the tightly coiled nucleocapsids with trypsin identified a major N-terminal cleavage product of NP (48 kDa) that remained associated with the RNA. The 12 kDa cleavage product was not identified, but it did not remain associated with the nucleocapsid (Heggeness et al., 1981). Additional cleavage of the 48 kDa product was observed in loosely coiled nucleocapsids, generating N-terminal 34 kDa and 15 kDa products, both associated with the template. A 12 kDa could be identified and it was shown to be derived from the 15 kDa product. None of the digested nucleocapsids appeared morphologically different from the native nucleocapsids when viewed with the electron microscope. The RNA in the native

and trypsin-digested nucleocapsids, in either conformation, was shown to be resistant to ribonuclease.

Taken together these data suggested that the domains of the NP protein responsible for assembling into ribonuclease resistant helical nucleocapsids, including the NP-NP and NP-RNA domains, are in the N-terminal 48 kDa fragment. Also these results indicated that the C-terminal ~100 aa are exposed to the surface and are not required for these interactions. Sequence analysis of the NP gene has identified aa 410 as a probable site for the first cleavage that generates the N-terminal 48 kDa fragment (Shioda et al., 1983; Morgan et al., 1984; Mountcastle et al., 1970; Buchholz et al., 1993), and aa 295 or 297 as possible sites for the cleavage that produces the N-terminal 34 kDa and 15 kDa fragments. Epitope mapping with monoclonal antibodies has identified four domains on the NP protein consisting of aa 289-295, aa 295-425, aa 425-455, and aa 455-524, with the majority of the antibodies tested recognizing epitopes in the two C-terminal domains (aa 425-524) (Deshpande and Portner, 1984; Gill et al.; 1988, Fisher, 1990). These results also suggested that the C-terminal ~100 aa are on the surface of the NP protein.

The NP protein expressed alone from a cDNA self-assembles into nucleocapsidlike particles in the absence of any additional SV proteins or RNA (Buchholz et al., 1993). Except for being shorter and heterogeneous in length the nucleocapsidlike particles were identical in morphology to wt

nucleocapsids. The data also suggested that the nucleocapsidlike particles contained nonspecific RNA (Buchholz et al., 1993). Analogous results have been reported for the MV nucleocapsid protein (N) expressed in animal cells (Spehner et al., 1991). In contrast, self-assembled MV N isolated from insect cells did not appear to contain RNA, based on their density (1.28 gm/cc), yet they appeared morphologically identical to wt MV nucleocapsids (Fooks et al., 1993). These results unambiguously identified NP-NP interactions and we would propose, as one model, that there are two binding faces, one which binds to the preceding NP protein and another which binds to the following NP protein.

A panel of 22 NP deletion mutants which covered the entire NP protein were assayed for both self-assembly and RNA replication (Buchholz et al., 1993; Curran et al., 1993). The data suggested that the first 400 aa were required for both self-assembly of the NP protein and DI-H replication *in vitro* (Buchholz et al., 1993; Curran et al., 1993). All deletion mutants except those of aa 400-415, 414-439, 426-497, or 456-524 were inactive in RNA replication (Curran et al., 1993), whereas the latter mutants all supported self-assembly and replication.

While the C-terminal 124 aa of the NP protein were not required for self-assembly or encapsidation of the nascent RNA, they were required for *in vivo* replication (Curran et al., 1993). Mutant NP proteins with deletions of aa 400-415,

414-439, or 456-524 were completely inactive in replication *in vivo* and deletion of aa 497-524 reduced the level of replication to 30% of wt NP protein. These data showed that the residues between 400-496 were required for the progeny nucleocapsids, with mutant proteins encapsidating the RNA, to serve as a template during amplification of the RNA genome and that removal of just the C-terminal 28 aa severely inhibited template function (Curran et al., 1993).

Mapping the domain for the NP-P interaction is not as straightforward since two types of NP-P complexes are formed, the soluble NP-P complex and the P-nucleocapsid (P-Nuc) complex. As discussed earlier, the NP protein requires coexpression of the P protein to provide the NP-P complex for activity for RNA replication. Of the 22 NP deletion mutants, only the deletion mutant with aa 456-524 removed was tested for dependence on the coexpression of the P protein and shown to be dependent on the coexpression of P for activity in RNA replication. These data suggest that this deletion mutant was capable of forming the soluble NP-P complex, which was used as the substrate for encapsidation. We would propose the three remaining C-terminal deletion mutants are also dependent on the coexpression of the P protein for activity in replication *in vitro*, suggesting that the N-terminal 400 residues contain the binding site required for the soluble NP-P complex.

To identify the P-Nuc binding site on the NP protein Buchholz et al. (1994) expressed the four NP mutants with the

deletions from aa 400 to 524. Nucleocapsidlike particles were formed by each of these mutants, which were isolated and tested for P binding. The NP proteins containing deletions of aa 400-415 or 414-439 bound the P protein, but deletion of aa 426-496 or 456-524 abolished the P-Nuc interaction. These data suggest that while the residues between 400-439 are required for template function they are not required to bind the P protein. The Nuc binding site for the P protein is within aa 439-524, but the exact boundaries are not clear, since replacing aa 426-496 restored 30% of the template function.

In summary, these data suggested that the domains for NP-NP, NP-RNA, and the soluble NP-P complexes are within the N-terminal 400 aa. The P-Nuc domain partially overlaps with the region of NP required for template function in the C-terminal 124 aa, suggesting other interactions or functions may map to this region of the NP protein (Buchholz et al., 1994). The N-terminal 400 aa are largely hydrophobic in character with a few clusters of charged residues (aa 60-71, 107-116, 121-129, 391-400) (Shioda et al., 1983; Morgan et al., 1984; Buchholz et al., 1993; Curran et al., 1993). Two of the charged clusters have been postulated to be RNA-binding domains (aa 60-72 and 107-115), although there is no data to support this assignment. However, the basic nature of the majority of these charges could lead to an interaction with the phosphates of the RNA (Morgan et al., 1984).

We propose that individual domains exist in the N-terminal 400 aa of the NP protein for the NP-P, NP-NP, and NP-RNA interactions. In order to map these domains two approaches will be taken. One utilizes a modified charge-to-alanine site-directed mutagenesis approach to target residues likely to be on the surface of the protein; therefore, potential sites for protein-protein or protein-RNA interactions (Cunningham and Wells, 1989; Bass et al., 1991; Bennett et al., 1991; Wertman et al., 1992). Two, to identify the domain of the NP protein required for self-assembly into nucleocapsidlike particles, regions of the NP protein will be isolated and fused to a monomeric soluble protein. Sedimentation analysis of the fusion proteins will identify protein-protein interactions as rapidly sedimenting complexes.

CHAPTER 2
MATERIALS AND METHODS

Cells and Viruses

Human lung carcinoma cells (A549 cells, American Type Culture collection) were maintained in a monolayer at 37°C with 5% CO_2 in F11 medium (GIBCO BRL) supplemented with 1 mM sodium pyruvate (100 mM, Mediatech), 2 mM L-glutamine (200 mM, Mediatech), 1% non-essential amino acid solution (100X, Mediatech), 1% penicillin-streptomycin (5000 I.U./ml and 5000 mcg/ml, respectively, penicillin-streptomycin, Mediatech), and 8% fetal bovine serum (FBS, GIBCO BRL). Vero cells (American Type Culture collection) were maintained as for A549 cells except the FBS was reduced to 5%.

Wild type (wt) Sendai virus (SV, Harris strain) was propagated in the allantoic fluid of 9-day old embryonated chicken eggs. The eggs were maintained at 37.4°C in an egg incubator and automatically turned every 30 min. The amount of Sendai virus that was used to inoculate the eggs was empirically determined, and the virus was grown at 33°C at 85% humidity for 3 days. The allantoic fluid was collected after placing the eggs at 4°C for 16 h then at -10°C for 10 min before harvesting and clarified by centrifugation at 2000 rpm for 10 min at 4°C. The virus was aliquoted and stored at -70°C. Sendai virus defective interfering particle, DI-H,

(Harris strain) was grown as described above and the amount of WT SV and DI-H used for co-infection was empirically determined for each infection.

The viruses were purified by pelleting through 15 ml 25% (v/v) glycerol in HNE buffer (10 mM HEPES, [*N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid], pH 7.4, 100 mM NaCl, 1 mM ethylenediamine tetraacetate [EDTA]) in an SW28 rotor at 26,000 rpm for 5 h at 4°C. The virus pellet was resuspended in 900 µl ET buffer (10 mM Tris-hydrochloride [Tris-HCL], pH 7.4, 1 mM EDTA) containing 10% dimethylsulfoxide (DMSO) (ET/DMSO). The virus was sonicated twice for 20 sec each and purified by banding on 7-60% (w/w) sucrose gradients in HNE buffer in an SW41 rotor at 24,000 rpm for 17 h at 4°C. The virus bands were collected, diluted with ET buffer, and pelleted in an SW41 rotor at 30,000 rpm for 2 h at 4°C. The virus pellet was collected in 200 µl ET/DMSO and stored at -70°C. The protein concentration of purified virus was determined by the Bradford method (Bradford, 1976) as outlined in *Current Protocols in Molecular Biology* (Ausubel et al., 1987).

Recombinant vaccinia virus containing the gene for phage T7 RNA polymerase (VVT7) (Fuerst et al., 1986) was kindly provided by Dr. Edward Niles (Suny; Buffalo, N.Y.) and grown in Vero cells. Briefly, a monolayer of Vero cells was grown in twenty 10 cm dishes (approximately 4 x 10^7 cells) at 37°C and the cells were infected with VVT7 at an multiplicity of infection (m.o.i.) of 0.05 pfu/cell. The cells were

harvested at 3-4 days post-infection (p.i.) by scraping the cells and medium with a rubber policeman. The cells and free virus were pelleted in a J10 rotor at 7000 rpm for 30 min at 4°C. The pellets were resuspended in 20 ml phosphate buffered saline (1.5 mM potassium phosphate monobasic, 4.3 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.2) supplemented with 1% penicillin-streptomycin (PBS + P/S) and the cells were disrupted by 2 freeze/thaw cycles followed by sonication for 60 sec. The virus titer was determined by a plaque assay on A549 cells and the virus was stored at -70°C.

Plasmids, Antibodies, and Probes

The plasmids pGEMSV-NP, pGEMSV-P, and pGEMSV-L, containing the SV nucleocapsid (NP), phosphoprotein (P), and large (L) genes, respectively, were provided by Dr. D. Kolakofsky (Geneva, Switzerland) (Curran et al., 1991). The plasmid pGSTSV-P containing the glutathione S-transferase (GST) gene fused in frame to the SV P gene (Horikami et al., submitted) was subcloned into pBSKS+ by S. Smallwood. The plasmids pBSMV-N and pBSMV-P containing the measles virus (MV) nucleocapsid (N) and phosphoprotein (P) genes, respectively, were provided by Dr. W. Bellini (CDC; Atlanta, GA). The plasmid pBSSV-NP (Chandrika et al., 1995) was constructed by subcloning the SV NP gene into pBSKS+ by Dr. R. Chandrika. All of the viral genes were cloned downstream of the phage T7 promoter.

The SV DI-H cDNA was cloned by C. Zack and S. Horikami into pSP65 (Promega). A cDNA of SV DI-H was obtained by

reverse transcription of nucleocapsid RNA isolated from A549 cells coinfected with SV and SV DI-H. The cDNA was amplified using recombinant polymerase chain reaction (PCR) and the amplification was done in two parts creating two arms (A and B) as previously described (Calain et al., 1992). The B arm was cloned into the plasmid vector pSP65 (Promega) using the XbaI and BamHI sites (pSP65-DIB). The hepatitis delta virus ribozyme and T7 terminator were provided by A. Ball (University of Alabama) and subcloned downstream of the B arm using the SmaI site (pSPDIB/Ribo/Term) (Calain and Roux, 1993). A full length DI-H clone (pSPDI-H) was created by subcloning the A arm (containing the T7 promoter) upstream of the B arm in pSPDIB/Ribo/Term using the XbaI site. The sequence of the DI-H clone was confirmed by restriction endonuclease mapping and double stranded (ds) dideoxy sequencing (Sequenase version 2.0 DNA sequencing kit, United States Biochemical).

Immunoprecipitation and immunoblotting utilized the following antibodies: a rabbit anti-Sendai virus antibody (α-SV) (Carlsen et al., 1985), a rabbit anti-SV L (α-L) antibody which is specific for the SV L protein (Horikami et al., 1992), and a rabbit anti-maltose-binding protein antibody (α-MBP) (Horikami et al., 1994).

A [α^{32}P]dCTP (50 μCi, 3000 Ci/mmole, Dupont, NEN Research Products) labeled random-primed SV DI-H DNA probe was produced using random hexamers ($pd(N)_6$, Pharmacia) based on the methodology of Feinberg and Vogelstein (1983).

Construction of NP Mutants

Site-Directed Mutagenesis using Recombinant PCR

Charge-to-alanine mutagenesis targeted two regions of the NP gene, from amino acid (aa) 107 to 129 and aa 362 to 375, generating 10 mutant NP genes containing 1 to 3 alanine substitutions each as shown in Figures 1 and 10. Recombinant polymerase chain reaction (PCR) was used to create the mutants NP108, NP111, NP114, NP121, NP126, NP362, NP364, NP370, and NP373 using two overlapping complementary mutagenic oligonucleotide primers and two standard outside oligonucleotide primers by the method of Higuchi et al. (1988). For screening of possible mutant clones, each mutagenic primer introduced a restriction endonuclease site that was created by two or more adjacent alanine substitutions or by the introduction of a separate silent mutation. The mutagenic oligonucleotides (restriction sites are underlined) used are shown in Table 1.

The mutants were created using two step PCR amplification with pGEMSV-NP as template. Except for the mutant NP370, the template for the first amplification was circular plasmid DNA and for NP370, the template was linearized to reduce mispriming. Each standard outside primer was paired with one of the mutagenic primers and PCR amplification generated a right and left arm that were gel purified (see below). The right and left arms were denatured separately for 3 min at 94°C then mixed while maintaining a temperature of 50°C or greater, and reannealed for at least

Table 1. Oligonucleotide primers

Mutant	Mutagenic primers[a]	Enzyme
NP108	SM212(+)-CATAGAGGCGGCGCCTAAGAGGACG	NarI
	SM213(-)-CCTCTTAGGCGCCGCCTCTATGTTG	
NP111	SM214(+)-GACCCTGCGGCGACGAAGACAGAC	BsoFI
	SM215(-)-TGTCTTCGTCGCCGCAGGGTCTTTCTC	
NP114	SM216(+)-AGGACGGCGACAGCCGGCTTCATTGTGAAGACG	NaeI
	SM217(-)-AATGAAGCCGGCTGTCGCCGTCCTCTTAGG	
NP121	SM218(+)-GTGGCGACCGCGGCTATGGAATATGAGAGG	SacII
	SM219(-)-TTCCATAGCCGCGGTCGCCACAATGAATCCGTC	
NP126	SM220(+)-GATATGGCATATGCGGCGACCACAGAATGG	NdeI
	SM221(-)-TGTGGTCGCCGCATATGCCATATCTCTGC	
NP362	SM291(+)-GGAAATGGCCTTACTTGGCCAAGCC	BalI
	SM294(-)-GGCTTGGCCAAGTAAGGCCATTTCC	
NP364	SM290(+)-GTTCTTAGCAGCACAAGCCGTG	BsoFI
	SM293(-)-CGGCTTGTGCTGCTAAGAAC	
NP370	SM209(+)-CGTGGCCGCGGCTGCTGAATCG	SacII
	SM208(-)-CAGCAGCCGCGGCCACGGCTTG	
NP373	SM289(+)-GATGCTGCTAGCGCGATCACGAGTGCC	NheI
	SM292(-)-CGTGATCGCGCTAGCAGCATCC	
NPΔ334-339	SM084(+)-ATTATCCTGCACTATGGGCCGTCGTACAGAACAA	none
	SM085(-)-TTGTTCTGTACGACGGCCCATAGTGCAGGATAAT	
NP146-6	SM146(+)-CTACAACATAGCGAAAGACCC	none

Mutant	Standard outside primers[b]
NP108, NP111, NP114, NP121, and NP126	SM030(+)-TAATACGACTCACTATAG
	SM123(-)-GGGAGCTCTGGGGCC
NP370	SM180(+)-GCCATGGCTTACAGTAG
	SM089(-)-CCCCTAGCGTCCTGGTCC
NP362, NP364, NP373, and NPΔ334-339	SM090(+)-GGTTGAGACCCTTGTGAC
	SM089(-)-CCCCTAGCGTCCTGGTCC

[a,b]The plus and minus symbols in parentheses following the primer number refers to the messenger sense (+) or genomic sense (-) of the oligonucleotide and the sequences are written 5′→3′. The restriction sites used for cloning and/or screening are underlined.

3 h at 50°C. The reannealed DNA was then PCR amplified using the two standard outside primers.

All PCR amplifications were done using the GeneAmp PCR System 9600 (Perkin Elmer) and 26 cycles of denaturation (50 sec, 94°C), annealing (50 sec, 42°C for all mutants except NP362, NP364, and NP373, which required 45°C to reduce mispriming), and extension (1 min 20 sec, for DNA products > 1000 base pairs (bp) or 60 sec for DNA products < 1000 bp, 72°C). The reactions (100 µl) contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 200 µM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 2 µM primer, and 0.5 U of *Taq* polymerase (Promega). The final PCR fragment was precipitated in 0.83 M ammonium acetate and 2 volumes 95% ethanol. The precipitated DNA was pelleted at 13,000 rpm for 15 min at 4°C and dried in a Speedvac (Savant).

The PCR products were cloned such that the PCR generated DNA sequences containing the mutations were substituted for the wild type sequence in pGEMSV-NP as follows. The PCR products for NP108, NP111, NP114, NP121, and NP126 were digested with EcoRI and AflII (nucleotides [nts] 1 to 629 of the NP sequence, strain Fushimi [F strain], accession number X17218) and the PCR products for NP362, NP364, NP370, and NP373 were digested with BstXI and EcoRV (nts 833 to 1339). The pGEMSV-NP plasmid was digested with both sets of enzymes in separate reactions followed by dephosphorylation with shrimp alkaline phosphatase (United States Biochemical), per manufacturer's protocol. The digested DNAs were separated on

a 1% (w/v) agarose gel (Seakem LE agarose) in TBE buffer (89 mM Tris-HCl, 2 mM EDTA, 89 mM boric acid) and the DNA was stained by adding ethidium bromide (0.1 µg/ml) to the running buffer. The DNA was visualized using a long wave UV lamp-366 NM (model UVL-56, Ultra-violet products, Inc.) and the DNA was excised using a #11 scalpel blade. The DNA was purified using a glassmilk silica matrix (The GENECLEAN II Kit, BIO 101 Inc.), per manufacturer's protocol.

The purified PCR products were subcloned into pGEMSV-NP at the same restriction sites by ligating the DNA for 12-16 h at 16°C. High concentration T4 DNA Ligase (2000 U, New England Biolabs) was used for all the ligation reactions in 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 µg/ml bovine serum albumin (BSA). The ligated products were transformed into competent ($CaCl_2$) E. *coli* TG1 cells (*K*12 Δ(*lac-Pro*AB) *Sup* E *thi hsd*Δ5/F' *tra*D36 *pro*AB+ *lac*I^q *lacZ*ΔM15) (Maniatis et al., 1989) and the transformation mix was plated on Luria broth (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) plates containing ampicillin (100 µg/ml) (LB + amp) at 37°C. The ampicillin-resistant colonies were screened by direct PCR amplification using the standard outside primers in a reaction volume of 25 µl. A portion of the colony was spotted onto a LB + amp replica plate before resuspending in the PCR reaction buffer. The PCR amplification was as above with an additional 5 min denaturation at 94°C before beginning the three step amplification cycle. The PCR products containing the correct

size fragment were precipitated in 0.83 M ammonium acetate and 2 volumes 95% ethanol.

The precipitated DNA was pelleted, dried, and digested with the appropriate restriction enzyme for screening. The digests, except the BsoFI digests, were separated on a 1% (w/v) agarose gel in TBE buffer. The BsoFI digests were separated on a 2.5% (w/v) Metaphor agarose gel (FMC BioProducts) in TBE buffer for resolution of the smaller fragments. Alkaline lysis plasmid preparations and CsCl purification (Maniatis et al., 1989) of the colonies that contained the proper size PCR product and restriction endonuclease pattern were done. Each clone was confirmed by the presence of the new restriction endonuclease site and by ds dideoxy sequencing of the entire fragment that was subcloned into pGEMSV-NP.

Oligonucleotide-Directed Mutagenesis using Phagemid DNA

The mutant NP107 containing a single charge-to-alanine substitution at aa 107 of SV NP was created using the Oligonucleotide-directed *in vitro* mutagenesis system version 2.1 kit (Amersham) per manufacturer's protocol. The protocol requires single-stranded phagemid DNA that can be isolated using Bluescript (pBSKS$^+$, Stratagene) vectors, but not pGEM (Promega) vectors. Therefore, the pBSSV-NP plasmid was used to generate the NP mutant at aa 107 (pNP146-6) and a DNA fragment containing the mutation was subsequently subcloned into pGEMSV-NP (NP107).

The methodology for growing the helper phage and producing single-stranded DNA was adapted from the protocols outlined by Vieira and Messing (1987). An aliquot of M13K07 helper phage, kindly provided by D. Hassett (University of Florida), was used to generate a high titer stock of M13K07 (2.2×10^{10} pfu/ml). The helper phage M13K07 was streaked onto a B plate (1% bacto-tryptone, 0.8% NaCl, 20% glucose, 1% thiamine, 1.5% bacto-agar) and overlaid with 4 ml soft top B agar (bacto-agar decreased to 0.6%) containing 0.5 ml of an E. *coli* JM101 (Δ(*lac-ProAB*)*Sup* E thi F' *tra*D36 *ProAB*+ *lacI*^{q} *lacZ*ΔM15) culture (OD_{600} >0.8) in LB and incubated at 37°C for 6.5 h. The top agar was poured from the most dilute side of the streak to the most concentrated side. Single small plaques were picked and inoculated into 3 ml LB containing kanamycin (70 µg/ml) and incubated for 16 h at 37°C. The culture was pelleted at 14,000 rpm for 5 min at 4°C and the supernatant was collected and repelleted as above. The final supernatant (M13K07 stock) was stored at 4°C. The M13K07 stock was titered on B plates using 100 µl of serially diluted M13K07, 200 µl log phase (OD_{600} = 0.430, ~1×10^8 cfu/ml) JM101 cells, and overlaid with 4 ml of soft top B agar. The plaques produced at 37°C were counted following a 7.5 h incubation.

Single-stranded DNA was produced as follows: early log phase cultures (6 ml, OD_{600} ~ 0.200) of bacteria containing pBSSV-NP LB + amp were superinfected with M13K07 at an m.o.i. of 10 and incubated in a shaker at 200 rpm for 1.25 h at

37°C. The cells were diluted (OD_{600} < 0.2) with LB + amp supplemented with kanamycin (70 µg/ml) and incubated in a shaker at 300 rpm for 14-18 h at 37°C. The cells were pelleted at 11,000 rpm for 10 min at 4°C. The phage particles in the supernatant were precipitated with 0.25 volume of 20% PEG/NaCl (20% (w/v) polyethylene glycol 8000, 2.5 M NaCl) and incubated for 30 min at 4°C. The phage particles were pelleted at 11,000 rpm for 10 min at 4°C. The pellet was resuspended in 200 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), extracted twice with 200 µl Tris-HCl-saturated phenol, pH 7.5, and chloroform (3:1), and once with 200 µl chloroform. The single-stranded DNA was precipitated in 0.3 M sodium acetate and 2 volumes of 95% ethanol.

The precipitated DNA was pelleted in a microfuge at 13,000 rpm for 15 min at 4°C, and the pellet was dried in a Speedvac. The pellet was resuspended in TE buffer and stored at -20°C. The yield of ss pBSSV-NP was approximately 60 µg. The mutagenic oligonucleotide is shown in Table 1 and the mutants and a plasmid preparation of the pNP146-6 mutant was made as described above. The mutation was confirmed by ds dideoxy sequencing.

For subcloning pNP146-6 to create NP107, a KpnI-AflII DNA fragment containing nt 1-629 of the mutant pNP146-6 was substituted for the wild type sequence in pGEMSV-NP at the same restriction sites. The KpnI-AflII cut pGEMSV-NP was dephosphorylated with shrimp alkaline phosphatase and the digested DNAs were gel purified, ligated, and transformed

into *E. coli* TGI cells, as described above. The mutation in pNP107 was confirmed by ds dideoxy sequencing.

Insertion and Deletion Mutants

Two NP mutants, NPHIII and NPΔ334-339, were created by C. Zack using either linker insertion (SalI linkers) or by recombinant PCR mutagenesis as follows: each mutant was created by first subcloning the complete 1.7-kb KpnI-BamHI gene fragment (nt 1-1639) of SV-NP from pGEMSV-NP into a KpnI-BamHI cut pGEM3ZF(+) vector that had been modified such that the SacI, SalI, AccI, HincII, PstI, SphI, and HindIII sites were deleted in the multiple cloning region (p*GEM3ZFSV-NP). For pNPHIII, the p*GEM3ZFSV-NP plasmid was cut with HindIII at nt 947 in the NP gene, blunt ended by filling in with T4 polymerase, followed by ligation of the SalI linkers(GGTCGACC). This insertion created a mutant NP gene that codes for an additional 4 aa (V, D, Q, and L) inserted between aa 296 and aa 297.

The deletion mutant pNPΔ334-339 was created by recombinant PCR as described previously using p*GEM3ZFSV-NP as template and the primers SM084 and SM089 to make the right arm and SM085 and SM090 to make the left arm (see Table 1 for primer sequences). The two arms were hybridized and amplified using the outside primers. The PCR product was cut with SacI and XbaI and gel purified. The purified 0.3-kb SacI-XbaI cut PCR fragment was cloned into a gel purified, SAP treated, and SacI-XbaI cut p*GEM3ZFSV-NP. This created a mutant NP gene that was missing nts 1064 to 1081; therefore,

had deleted the codons for the residues from 334 to 339 (S, Y, A, M, G, and V).

The mutant DNAs were transformed into *E. coli* UT481 (met$^-$ thy$^-$ Δ(*lacPro*)r$^-$m$^-$ *Sup* D TN10/F' *tra*D36 *pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15) cells and plasmid preparations made as outlined above. The insertion mutation at aa 296 was confirmed by digestion at the new SalI restriction site in pNPHIII and both the insertion and deletion mutations were confirmed by ds dideoxy sequencing of individual clones.

Random-Primed Site-Directed Mutagenesis

The mutants NP260-1, NP299-5, NP313-2, NP324-1 and NP324-5 were constructed by C. Zack using PCR-directed mutagenesis and cloning the PCR fragment into the pCR II vector (TA Cloning Kit, Invitrogen). The mutation was randomly introduced at one or two conserved hydrophobic aa downstream of a unique restriction site in the NP gene and the primer was designed to include the restriction site as well as the mutation (see Table 2). The same standard downstream primer (SM089) was paired with each of the mutagenic primers for amplification and after cloning the PCR fragment into pCR II (pCRIINP) the mutations were identified by ds dideoxy sequencing. The mutant NP fragment in the pCR II vector was subsequently subcloned by A. Pieters into p*GEM3ZFSV-NP. This was done by replacing the wt NP sequence in p*GEM3ZFSV-NP with the corresponding fragment from the mutant pCRIINP plasmids (Table 2) and the ligated DNAs were transformed into *E.coli* UT481 cells. Alkaline lysis

Table 2. Site-directed mutations in the central conserved region

Mutant	Mutagenic primer[a]	Enzyme
NP260-1	SM112(+)-CATCCAGATAGTTGGGAACNACNTCCGAGA	BstXI
NP299-5	SM110(+)-TAATAAGCTTAGAAGCNTCNTAGAGACC	HindIII
NP313-2	SM109(+)-CCCAGAGCTCCCTTTNTCTGTNTCCTC	SacI
NP324-1	SM108(+)-CTCAAGGACCCTGTTCATGGTGAANTTGCT	PpuMI
NP324-5	SM108(+)-CTCAAGGACCCTGTTCATGGTGAANTTGCT	PpuMI

Mutant	Standard outside primer[b]
NP260-1, NP299-5, NP313-2, NP324-1, and NP324-5	SM089(-)-CCCCTAGCGTCCTGGTCC

Mutant	Nucleotide changes	Amino acid changes	Gene fragments subcloned[c]
NP260-1	T842→G	Y260D	0.506-kb BstXI-EcoRV (nts 833-1339
NP299-5	C959→A, A962→G	L299I and I300V	0.392-kb HindIII-EcoRV (nts 947-1339)
NP313-2	A1001→T	I313F	0.400-kb SacI-EcoRV (nts 994-1339)
NP324-1	T1034→G	F324V	0.324-kb PpuMI-EcoRV (nts 1015-1339)
NP324-5	T1034→A	F324I	0.324-kb PpuMI-EcoRV (nts 1015-1339)

[a,b]The plus and minus symbols in parentheses following the primer number refers to the messenger sense (+) or genomic sense (-) of the oligonucleotide and the sequences are written 5′→3′. The bold nucleotide, **N,** was randomized during primer synthesis, incorporating an A, C, G, or T at that position. The restriction sites used for cloning and/or screening are underlined.
[c]All gene fragments were subcloned into the identical sites in p*GEM3ZFSV-NP.

preparations of each clone were made and the entire fragment that was subcloned into p*GEM3ZFSV-NP was sequenced as described above.

Maltose-Binding Protein-Viral Fusion Proteins

The plasmid pMBP containing the MalE gene (maltose binding protein, (MBP) was created by cloning a PCR fragment from the pMal-c2 vector into a pGEM3 vector missing the EcoRI site in the multiple cloning sequence (see Table 3). The PCR fragment contained the complete *MalE* gene other than it was lacking the signal sequence. The pMal-c2 vector was kindly provided by the R. Condit lab (University of Florida) and the sequence for the SM236 primer that contains a KpnI restriction site and the eukaryotic translational start sequence (AAAATGA) was kindly provided by the M. Swanson lab (University of Florida) (Anderson et al., 1993).

The fusions were created by cloning, in frame, PCR products of SV-NP gene fragments downstream of the MBP-coding region as described in Table 3. The digested DNAs were gel purified, ligated, and transformed into *E. coli* TG1 cells as described above and in Table 3. The ampicillin resistant colonies were screened by PCR amplification using the above primers and plasmid preparations of positive clones were made (Maniatis et al., 1989). The size and orientation of the NP inserts in the fusion constructs were confirmed by restriction endonuclease mapping and apparent molecular weight on SDS-PAGE.

Table 3. MBP-NP fusion protein primers and plasmids

Oligonucleotide and Sequence[a]	Enzyme
SM236(+)-CCCGGTACCAAAATGAAAATCGAAGAAGGTAA	KpnI
RM251(-)-GAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG	none
SM204(+)-CCAGGAATTCGGGAACTACATCCG	EcoRI
SM222(-)-ACATGGATCC**TCA**AAGGTATGTCCTCCCTG	BamHI
SM336(+)-CGATGAATTCAAGCTTAGAAGCCTC	EcoRI
SM273(+)-GTTCGGATCCGCCGGGTTGTTGAGC	BamHI
SM089(-)-CCCCTAGCGTCCTGGTCC	none
SM272(+)-GACAGGATCCGATATGGAAATGTTCTTACTAGG	BamHI
SM240(-)-TTGTCGTTAGAACGC	none
SM210(-)-AATAGGATCC**TCA**AAAGTAAGATCGGCC	BamHI
SM211(+)-ATGTGAATTCGATACATATATCGTAGAGGCAGG	EcoRI

Plasmid	Description
pMBP	A 1.2-kb KpnI-HindIII cut PCR fragment of pMal-c2 (primers SM236 and RM251) was subcloned into KpnI-HindIII cut pGEM3ΔRI.
pMBP.NP1	A 0.3-kb EcoRI-BamHI cut PCR fragment of SV-NP (primers SM204 and SM222) was cloned into EcoRI-BamHI cut pMBP.
pMBP.NP1-C	A 0.190-kb EcoRI-BamHI cut PCR fragment of SV-NP (primers SM336 and SM222) was cloned into EcoRI-BamHI cut pMBP.
pMBP.NP2	A 0.770-kb BstXI, blunt ended, BamHI cut fragment of SV-NP (primers SM273 and SM089) was cloned into PstI, blunt ended, BamHI cut pMBP.
pMBP.NP3A	A 0.880-kb BamHI-HindIII cut PCR fragment of SV-NP (primers SM273 and SM089) was cloned into BamHI-HindIII cut pMBP.
pMBP.NP3B	A 1.1-kb BamHI cut PCR fragment of SV-NP (primers SM273 and SM222) was cloned into a BamHI cut pMBP.
pMBP.NP4	A 0.52-kb BamHI cut PCR fragment of SV-NP (primers SM272 and SM240) was cloned into a BamHI cut pMBP.
pMBP.NP295N	A 0.3-kb EcoRI-BamHI cut PCR fragment of pSV295MV (primers SM204 and SM210) was cloned into EcoRI-BamHI cut pMBP.
pMBP.N295NP	A 0.3-kb EcoRI-BamHI cut PCR fragment of pMV295SV (primers SM211 and SM222) was cloned into EcoRI-BamHI cut pMBP.
pMBP.N1	A 0.3-kb EcoRI-BamHI cut PCR fragment of pBSMV-N (primers SM211 and SM210) was cloned into EcoRI-BamHI cut pMBP.

[a]The plus and minus symbols in parentheses following the primer number refers to the messenger sense (+) or genomic sense (-) of the oligonucleotide and the sequences are written 5′→3′. Stop codons are indicated by bold lettering. The restriction sites used for cloning and/or screening are underlined.

Infection and Transfection

Subconfluent A549 cells in 60 mm dishes (approximately 4.8 x 10^6 cells) were infected with VVT7 at a m.o.i. of 2.5 pfu/cell for 1 h at 37°C in 0.5 ml Fll medium (GIBCO BRL) supplemented with 1% penicillin-streptomycin, 2 mM glutamine, 14 mM HEPES, pH 7.4 (Fll adsorption medium). At 1 h p.i. the inoculum was removed, the cells washed with unsupplemented Opti-MEM (GIBCO BRL) and replaced with 2 ml Opti-MEM supplemented as above. As indicated in the Figure legends, cells were transfected at 37°C with one or more CsCl purified plasmids containing individual genes cloned downstream of the phage T7 promoter using 10 µl lipofectin (Bethesda Research Laboratories) per 2.5 µg plasmid DNA in 1 ml Opti-MEM adsorption medium.

RNA Replication

In Vitro Replication

At 18 h post transfection (p.t.) cytoplasmic cell extracts were prepared at 4°C using lysolecithin (L-α-lysophosphatidylcholine, palmitoyl; Sigma Chemical Company) permeabilization (Peluso and Moyer, 1983). The dishes were placed on ice and the cells washed with 3 ml wash solution (150 mM sucrose, 30 mM HEPES, pH 7.4, 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate) and permeabilized with lysolecithin (250 µg/ml in wash solution, 1 ml) for 1 min with rocking. Wash solution (3 ml) was added, the liquid aspirated, and the cells drained for 3-4 minutes. The cells were then scraped with a rubber policeman into 100 µl Sendai virus reaction mix

(SV RM) containing 0.1 M HEPES, pH 8.5, 0.05 M NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 10% glycerol, 0.5 U/µl RNasin, 1 mM each dithiothreitol (DTT), spermidine, ATP, GTP, UTP and 10 µM CTP (Horikami et al., 1992). For unlabeled, that is cold reactions, the CTP concentration was increased to 1 mM. The cells were lysed by pipeting 20X and the nuclei pelleted at 1600 rpm for 5 min at 4°C in a microfuge.

The supernatant (cytoplasmic cell extract) was collected and the volume adjusted to 100 µl with additional SV RM, if necessary. Dactinomycin (20 µg/ml) was added to the cell extract after 10% of the cell volume was removed for immunoblot analysis. The remaining cell extract was mixed with 2.2 µg purified DI-H disrupted with 0.1% Triton X-100 (dd DI-H) for 10 min at 4°C and 50 µCi [α-^{32}P]CTP (3000 Ci/mmol; Amersham) and then incubated for 2 h at 30°C. Unencapsidated product RNA was digested with micrococcal nuclease (MN, 10 µg/ml) in the presence of 1 mM $CaCl_2$ for 30 minutes at 37°C, and the MN was inactivated by the addition of 7.5 mM ethylene glycol-bis(*B*-aminoethyl ether)-*N,N*-tetraacetic acid, pH 8.0, (EGTA).

The nuclease resistant replication products were purified by banding on 5 ml cesium chloride (CsCl) gradients prepared by layering from the bottom 2 ml 40% (w/w) CsCl in TNE (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA), 2 ml 20% (w/w) CsCl in TNE, and 0.9 ml 30% (v/v) glycerol in 10 mM HEPES, pH 8.5, in an SW55 rotor at 36,000 rpm for 16 h at 4°C. It has been shown previously that the SV nucleocapsids

band at the 20-40% interface, i.e., at a density of 1.29 gm/cc to 1.33 gm/cc (Carlsen et al., 1985; Chandrika et al., 1995; Buchholz et al., 1993; Kolakofsky, 1976). This nucleocapsid containing region of the individual gradients was collected, diluted in ET buffer, and pelleted in an SW55 rotor at 50,000 rpm for 2 h at 4°C (Horikami et al., 1992). The pellet was collected in 300 µl 1X NENSH (100 mM NaCl, 50 mM magnesium acetate, pH 5.2, 10 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]), containing 250 µg/ml proteinase K (Merck), 250 U/ml heparin, 16 µg/ml tRNA and incubated for 1 h at 37°C. The sample was extracted twice with 10 mM HEPES-saturated phenol, pH 7.4, and chloroform (1:1), followed by precipitation of the RNA in 0.3 M NaCl and 2.5 volumes 95% ethanol.

The RNA was pelleted in a microfuge at 13,000 rpm for 15 min at 4°C and the pelleted RNA was dried in a Speedvac. The RNA was resuspended in 45 µl sample buffer (2.5 mM citrate buffer, pH 3.5, 6 M urea, 20% sucrose, 5 mM EDTA, 0.012% bromphenol blue), denatured by boiling for 2 min and quenching on ice. The RNA was analyzed by electrophoresis on a horizontal (22 cm/180 ml) 1.5% agarose-6 M urea gel (Wertz and Davis, 1979). Electrophoresis was set at 150 volts (V) until the RNA samples had entered the gel and then the voltage was decreased to 90-100 V overnight at 4°C (1760 V-h). The gel was processed for fluorography by washing 5 times in 400 ml 7% acetic acid at 20 min intervals, dehydrating 2 times in 400 ml 95% ethanol at 30 min

intervals, impregnating with 400 ml 5% PPO (2,5 diphenyloxazole; Sigma Chemical Co.) in acetone for 45 min, and hydrating the gel in water for at least 1 h (Baker and Moyer, 1988). The gel was dried for 1.5 h at 60°C followed by 1.5 h at 72°C and exposed to Kodak X-Omat film. A phosphorimager (Molecular Dynamics) was used to quantitate the replication products.

In Vivo Replication

In vivo replication was measured by cotransfecting pSPDI-H with the plasmids containing genes for the NP, P, and L proteins (see Figure legends). Transcription of the DI-H clone by T7 polymerase generated a full-length plus-sense DI-H RNA and the (+) DI-H RNA was encapsidated by the expressed NP protein. The (+)DI-H RNA-NP was then replicated by the expressed NP, P, and L proteins (Curran et al., 1993). DI-H RNA replication with the *in vivo* assembled templates was measured either by making cell extracts and assaying replication by *in vitro* DI-H RNA synthesis or by northern analysis of the RNA extracted from the cell.

For *in vitro* DI-H RNA synthesis, infection and transfection were at 37°C and cell extract preparation was as described for *in vitro* replication. RNA synthesis was detected by adding 50 µCi [α-^{32}P]CTP and incubating for 2 h at 30°C. The nuclease resistant replication products were banded on CsCl, the RNA was purified, analyzed by gel electrophoresis, and the product DI-H RNA quantitated as described for *in vitro* replication.

For northern analysis, the infection and transfection was as described above except 100 mm dishes (approximately 1.8 x 10^7 cells) were used and the volumes adjusted as follows: VVT7 infection was in 2.5 ml F11 adsorption medium, transfection was in 4 ml Opti-MEM adsorption medium, and the lipofectin/DNA mix remained in 1 ml Opti-MEM. Following transfection, the cells were incubated at 32°C or 37°C for 36 h or 22 h, respectively. Cell extracts were prepared as described above except the lysolecithin and the wash solution were increased to 2 ml and 5 ml, respectively, and the cells were scraped into 200 µl HNDG (0.1 M HEPES, pH 8.5, 0.05 M NH_4Cl, 1 mM DTT, 10% glycerol). The replication products were digested with MN, banded on CsCl gradients, proteinase K treated, phenol-chloroform extracted and ethanol precipitated as described for *in vitro* replication.

The precipitated product RNAs were pelleted in a microfuge at 13,000 rpm for 15 min at 4°C and dried in a Speedvac. The dried pellets were resuspended separately in 15 µl sample buffer, denatured and separated on a horizontal (10 cm/40 ml) 1.5% agarose-6 M urea gel for 200 V-h at room temperature. The gel was washed 5 times at 20 min intervals in 2X SSC (0.3 M NaCl, 30 mM sodium citrate) and the RNA was transferred by capillary action for 24 h at room temperature onto Hybond-N nitrocellulose paper (Amersham). The RNA was UV crosslinked (Stratalinker UV Crosslinker 1800, Stratagene) to the nitrocellulose and the nitrocellulose was kept moist

during the crosslinking by placing it on filter paper premoistened with 5X SSC.

The blot was incubated for 4.5 h at 42°C in 5 ml of boiled and quenched prehybridization buffer (50% formamide, 4X Denhardts, 0.05% SDS, 2.5X SSPE [0.9 M NaCl, 2.5 N NaOH, 5 mM EDTA, 50 mM sodium phosphate, pH 7.4], 200 µg/ml salmon sperm DNA) in a plastic bag. Denatured [α-^{32}P]dCTP (3000 µCi/mmole, Dupont, NEN Research Products) labeled DI-H probe (3-6 x 10^6 cpm) was added to the prehybridization solution and incubated for 16 h at 42°C. The blot was washed 3 times in 1X SSC/0.5% SDS for 5 min at room temperature, followed by 2 washes in 0.1X SSC/0.1% SDS for 15 min at 50°C. The blot was exposed to Kodak X-Omat film and the product RNA quantitated on the phosphorimager.

Protein Analysis

Protein Synthesis *In Vivo*

For steady state labeling, A549 cells (4.8 x 10^6) were infected with VVT7 and transfected with the plasmids as indicated in the Figure legends. At 5.5 h p.t. the medium was removed and the proteins labeled for various times, as indicated in the Figure legends, with 66 µCi/ml Tran^{35}S-label (ICN Pharmaceuticals, Inc.) in Dulbecco's minimal essential medium without L-glutamine, methionine, and cysteine (DMEM, Mediatech), containing one-tenth the normal methionine and cysteine (10% (v/v) Fll, 1% penicillin-streptomycin, 2 mM L-glutamine, and 14 mM HEPES, pH 7.4). Cell extracts were prepared in 300 µl SV RM salts (0.1 M HEPES, pH 8.5, 0.05 M

NH_4Cl 7 M KCl, 4.5 mM magnesium acetate) containing 0.25% NP40 and 1 µg/ml aprotinin and incubated for 20 min with rocking at 4°C. The cells were scraped using a rubber policeman, vortexed, and pelleted at 13,000 rpm for 30 min at 4°C. The supernatant was collected and analyzed by sedimentation on glycerol or CsCl gradients, by bead binding, and by immunoprecipitation.

For pulse-chase analysis of proteins, the infected and transfected cells were pulse-labeled for 30 min with Tran^{35}S-label (66 µCi/ml) at 5.5 h p.t. in methionine- and cysteine-free DMEM (1% penicillin-streptomycin, 2 mM L-glutamine, and 14 mM HEPES, pH 7.4). The medium was removed and cell extracts prepared immediately or at various times (see Figure legends) p.t. after a chase with medium containing 10-fold excess methionine and cysteine (0.5% 2X F11, 0.2% 50X MEM amino acids solution without L-glutamine [Mediatech], 48 mM NaOH, 1% penicillin-streptomycin, 2 mM glutamine, 14 mM HEPES, pH 7.4). The cell extracts were stored at -70°C or the proteins were analyzed by immunoprecipitation and SDS-PAGE.

The cells were Tran^{35}S-labeled for all assays except electron microscopy and immunoblot analysis of discontinuous CsCl gradients of wt and mutant NP proteins. In these cases the cells were infected and transfected as above and incubated for 18 h or 20 h at 37°C in the Opti-MEM adsorption medium.

Protein Synthesis *In Vitro*

The plasmids containing the genes coding for the MBP-viral fusion proteins were linearized downstream of the protein-coding region as follows: pMBP, pMBP.N1, pMBP.NP2 pMBP.NP3A, and pMBP.NP4 were cut with HindIII; pMBP.NP1 was cut with BamHI and pMBP.NP3B was cut with XbaI. The fusions truncated within the NP or N protein coding regions were digested as follows: pMBP.NP1-H was cut with HindIII, pMBP.NP1-B was cut with BsgI; pMBP.NP2-A was cut with AflII, pMBP.NP2-B was cut with BbsI, pMBP.NP2-P and pMBP.N1-P were cut with PflMI. The linearized DNAs were transcribed *in vitro* with purified recombinant T7 polymerase. The T7 transcripts were translated in a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-Methionine (NEN Dupont) according to the manufacturer's protocol. The following circular plasmids, pMBP, pMBP.NP1-C, pMBP.NP4, pMBP.NP1Δ334-339, and pMBP.NP1-HIII, were transcribed and translated *in vitro* using the TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of ^{35}S-Methionine (NEN Dupont) according to the manufacturer's protocol.

Glycerol Gradient Analysis

One half of the Tran^{35}S-labeled cell extracts and a [α-^{32}P]CTP labeled DI-H nucleocapsid product (RNA-NP) from an *in vitro* replication reaction were analyzed by sedimentation on separate 5-30% (v/v) glycerol gradients (12 ml, in SV RM salts) in an SW41 rotor at 36,000 rpm for 105 min at 4°C. Fractions (0.5 ml) were collected from the top and stored at

-70°C. The trichloroacetic acid-precipitable activity of the gradient fractions containing [α-^{32}P]CTP labeled DI-H product was determined. The Tran^{35}S-labeled proteins from each fraction (0.25 ml of each) were immunoprecipitated with an α-SV antibody as described below, separated by 9% SDS-PAGE (Laemmli, 1970), and quantitated on the phosphorimager. All of the protein gels were processed for fluorography by dehydrating in two washes of DMSO (200 ml) for 20 min at rt, impregnated with 20% PPO in DMSO for 45 min at rt, and hydrated in water for one hour at room temperature (rt).

Cesium Chloride Gradient Analysis

For CsCl gradient analysis of the proteins SV-NP, NP362, and NP370, A549 cells (4.8 x 10^6) were infected with VVT7 and transfected in duplicate. The duplicate Tran^{35}S-labeled cell extracts of each protein were combined and analyzed by sedimentation on separate linear 20%-40% (w/v) CsCl gradients (12 ml) in an SW41 rotor at 36,000 rpm for 16 h at 4°C. Fractions (0.5 ml) were collected from the top and stored at -70°C. The density of each fraction was determined with a refractometer and the proteins in each fraction (0.2 ml of each) were immunoprecipitated with an α-SV antibody as described below. The proteins were analyzed by 9% SDS-PAGE and the protein bands were quantitated on the phosphorimager.

For analysis of the amount of self-assembled wt and mutant NP proteins, all of the Tran^{35}S-labeled or unlabeled cell extracts were analyzed by sedimentation on separate CsCl step gradients (layered from the bottom; 1.4 ml 40% CsCl, 1.4

ml 30% CsCl, 1.4 ml 20% CsCl, 0.6 ml 30% glycerol in 10 mM HEPES, pH 8.5) in an SW55 rotor at 36,000 rpm for 16 h at 4°C (Buchholz et al., 1993). Fractions (0.714 ml) were collected from the top and stored at -70°C. The unlabeled or radiolabeled proteins in each fraction were analyzed by immunoblotting with an α-SV antibody (25 µl of each fraction) or by immunoprecipitation (0.2 ml of each fraction), respectively, and the density of individual fractions was determined with a refractometer. The immunoprecipitated proteins were separated by 9% SDS-PAGE and quantitated on the phosphorimager.

Glutathione-Sepharose Bead Binding

A549 cells (4.8×10^6) were infected as above and wt or mutant NP plasmids were transfected singly or together with pGSTSV-P. Cell extracts were prepared as described above in SV salts containing 0.25% NP 40 and one-fourth (75 µl) of the cell extract was used for bead binding and an identical aliquot (75 µl) was used for immunoprecipitation as described below. Glutathione-Sepharose 4B beads (15 µl per reaction, Pharmacia Biotech) were prepared by washing in SV RM salts two times followed by blocking for 15 min at 4°C in 1 ml of SV RM salts containing 0.1% NP40, 0.5% nonfat dry milk (NFDM), and 10 mg/ml BSA. The blocked beads were washed two times in SV RM salts and the final volume of the beads was adjusted with SV RM salts to 50 µl beads per reaction.

The Tran^{35}S-labeled cell extracts (75 µl) were incubated with the blocked beads (50 µl) for 15 min at 4°C. The beads

were washed three times with SV RM salts containing 0.25% NP40 and 1µg/ml aprotinin. All centrifugation was done in a mini-centrifuge (Costar). The beads were resuspended in 45 µl 2X lysis buffer (4% SDS, 55 mM Tris-HCl, pH 6.8, 185 mM DTT, 37% (v/v) glycerol, 0.01% bromphenol blue) and denatured by boiling for 2 min. The beads were pelleted for 3 min at rt and the supernatant was analyzed by 9% SDS-PAGE.

Immunoprecipitation

Cell extracts or glycerol gradient fractions were brought up to a final volume of 300 µl with SV RM salts containing 0.25% NP40 and 1 µg/ml aprotinin and preadsorbed with 100 µl *Staphylococcus aureus* (Cowan strain) (Carlsen et al., 1985) for 30 min at 4°C. The bacteria were pelleted at 13,000 rpm for 3 min at rt and the supernatant transferred to a new microfuge tube. The supernatant (antigen) was incubated with the appropriate antibody (see Figure legends) for 1.25 h at 4°C. The antigen-antibody complexes were collected with 100 µl *Staphylococcus aureus* (Cowan strain) for 30 min at 4°C. The precipitate was pelleted at 13,000 rpm for 1 min and 20 sec and the supernatant was removed using a fine-tipped pasteur pipet. The pellet was washed by resuspending in 800 µl of SV RM salts supplemented as above, pelleted 1 min and 20 sec, and the wash repeated. The pellet was resuspended in 45 µl of 1X lysis buffer, boiled for 2 min, and repelleted at 13,000 rpm for 3 min at rt. The supernatant was analyzed by 9% SDS-PAGE and the proteins quantitated on the phosphorimager.

For the CsCl gradient fractions, the following changes were made to the above protocol. The individual fractions were brought up to a final volume of 1 ml in 1% NP40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP40, 1 µg/ml aprotinin) (Huber et al., 1991). The preadsorption incubation was increased to 45 min, the antibody incubation was increased to 2.5 h, and the washes were with 1% NP40 lysis buffer (800 µl).

Sedimentation through 30% Glycerol

To determine the total protein, 1 µl (4%) of the *in vitro* transcription and translation reaction (25 µl total volume) was removed, diluted in 30 µl 2X lysis buffer, and stored at 4°C. To analyze protein-protein complex formation, 8 µl (32%) of the *in vitro* transcription and translation reaction was diluted in 132 µl of SV RM salts containing 0.25% NP40, and analyzed by pelleting separately through 30% (v/v) glycerol (5 ml) in an SW55 rotor at 50,000 rpm for 90 min at 4°C. The pellets were collected in 50 µl 2X lysis buffer and analyzed with the total protein samples by 9% SDS-PAGE and the proteins quantitated on the phosphorimager.

Immunoblot Analysis

For immunoblots of CsCl gradient fractions, equal volumes of 2X lysis buffer and the CsCl gradient fractions (25 µl) were mixed and denatured by boiling for 2 min. The proteins were separated on 10% mini-protean polyacrylamide-SDS gels and then transferred to nitrocellulose (nc) (Schleicher & Schuell) at 45 V for 16 h at 4°C using the

Mini-PROTEAN electrophoresis system (BioRad). The gel and nc were prepared for transfer by equilibration in two washes of transfer buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol) for 10 min at rt. Following transfer, the nc was incubated in blocking buffer (10% Newborn Calf Serum [GibcoBRL], 5% BSA in TBS plus Tween 20 [0.02 M Tris base, 0.5 M NaCl, 0.05% (v/v) Tween 20]) for 1 h at 45°C.

The proteins were identified with an α-SV antibody (1:250 dilution) for 1 h at rt followed by an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:3000 dilution in TBS, FisherBiotech, 0.2 mg/ml) for 1 h at rt. The nc was washed two times in TBS plus Tween 20 for 15 min at rt before and after each antibody incubation. The nc was incubated with the substrate solution NBT/BCIP (220 µl, nitroblue tetrazolium, 50 mg/ml in 70% dimethyl formamide, Promega/110 µl, 5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine Salt, 50 mg/ml in 100% dimethyl formamide, Fisher) in alkaline phosphatase buffer (20 ml, 100 mM Tris, pH 9.0, 100 mM NaCl, 5 mM $MgCl_2$), until color development was seen (approximately 1-2 min) and the reaction was stopped with water.

For the immunoblots on the *in vitro* and *in vivo* replication extracts, the above protocol was followed except in Figure 24. The MBP-NP fusion proteins were detected with an α-MBP antibody (1:250 dilution) following the α-SV antibody incubation. The washes were repeated between the

two primary antibody incubations and before adding the alkaline phosphatase-conjugated secondary antibody.

For the immunoblots on the extracts used in the northern analysis (Fig. 7B) the electrophoresis and transfer were as outlined above, but the antigen was detected using enhanced chemiluminescence (ECL, Amersham Life Science) per manufacturers protocol. Briefly, the nc was blocked in 5% (w/v) NFDM in PBS for 2 h at rt and incubated with an α-SV antibody (1:250) for 1 h at rt. The nc was washed twice for 5 min each and once for 15 min in PBS, and then incubated with horse radish peroxidase (HRPO)-conjugated goat anti-rabbit antibody (1:5000 dilution in PBS containing 0.5% NFDM, FisherBiotech, 0.5 mg/ml) for 1 h at rt. The blot was washed three times as above in PBS. The ECL reagents were mixed (1:1) and incubated with the nc for 1 min at rt (in the dark). The nc was wrapped in plastic wrap and exposed to Kodak X-Omat film for 2-3 sec.

SV Polymerase Binding to Self-Assembled Nucleocapsids

A549 (4.8×10^6) cells were infected with VVT7 and either transfected with no plasmids (mock), or with the wt or mutant NP plasmid (self-assembled nucleocapsids), or cotransfected with the SV-P and SV-L plasmids (polymerase) such that there was one P and L cotransfected dish for each mock, wt and mutant NP transfected dish. At 5.5 h p.t. the cells were pulse-labeled for 1 h (P and L co-transfections) or for 3 h (mock and self-assembled nucleocapsid transfections) with Tran^{35}S-label (66 μCi/ml) in 10%

methionine and cysteine DMEM supplemented as described for protein synthesis *in vivo*. The cells cotransfected with SV-P and SV-L were harvested using lysolecithin permeabilization and collected in 100 µl SV RM salts containing 1 mM ATP and 1 µg/ml aprotinin (Horikami and Moyer, 1995). The duplicate extracts containing the P and L proteins were combined after the nuclei were pelleted.

The cells mock transfected or transfected with wt or mutant NP were harvested in SV salts containing 0.25% NP40 and 1 µg/ml aprotinin (300 µl). The mock cell extract and the self-assembled wt or mutant nucleocapsids were purified by pelleting separately through 30% (v/v) glycerol (5 ml) in an SW55 rotor at 50,000 rpm for 90 min at 4°C and the pellets were collected in 75 µl SV RM salts. Equal aliquots (100 µl) of the polymerase extract were mixed with the purified mock or nucleocapsid preparations and incubated for 1 h at 30°C. As a positive control the polymerase extract was also incubated with polymerase-free purified DI-H. The reactions were pelleted through a step gradient containing 2.5 ml of 30% and 50% (v/v) glycerol in 10 mM HEPES, pH 8.5, in an SW55 rotor at 50,000 rpm for 90 min at 4°C. The pellets were resuspended in 1% NP40 lysis buffer (250 µl) and the proteins were immunoprecipitated with the α-SV and α-L antibodies and separated on a 7.5% polyacrylamide-SDS gel.

Electron Microscopy

For electron microscopy of the self-assembled wt and mutant NP370 proteins, A549 cells (4.8×10^6) were infected

with VVT7 and transfected in duplicate. The duplicate unlabeled cell extracts for each protein were combined and pelleted through 30% (v/v) glycerol (5 ml) in an SW55 rotor at 50,000 rpm for 90 min at 4°C. The pellets were collected in 75 µl ET buffer and analyzed by negative staining with 2% uranyl acetate by the Electron Microscopy Core Laboratory in the Interdisciplinary Center for Biotechnology Research at the University of Florida.

CHAPTER 3
TEMPLATE FUNCTION

Introduction

The nucleocapsid protein (NP, 524 aa) of Sendai virus is an essential component of both protein-protein and protein-RNA interactions (NP-NP, NP-P, NP-RNA, and P/L-Nuc) required for viral RNA replication (Horikami et al., 1992; Buchholz et al., 1993; Curran et al., 1993). Only encapsidated RNA is recognized by the viral polymerase (Emerson and Wagner, 1972, Buchholz et al., 1993) suggesting that the NP protein has functional as well as structural roles in RNA replication (Buchholz et al., 1993). Approximately 2600 molecules of the NP protein encapsidate the viral RNA genome and encapsidation of the viral RNA also renders the RNA nuclease resistant (Galinski and Wechsler, 1991; Moyer and Horikami, 1991).

Evidence presented by Curran et al. (1993) suggested that the N-terminal 400 aa of the NP protein are required for RNA replication *in vitro* since any deletion, even one as small as 7 aa, within the first 400 aa abolished activity. They found that the C-terminal tail (ca. 124 aa) of the NP protein was not required for encapsidation of the nascent RNA, but was required for replication *in vivo* suggesting that

the C-terminal amino acids were required for the progeny nucleocapsids to serve as a template during amplification of the RNA genome.

To identify residues within the first 400 aa of the NP protein required for various aspects of viral RNA replication, we selected clustered charge-to-alanine mutagenesis because of the likelihood of targeting surface residues and producing a number of stable mutant proteins which would exhibit a mutant phenotype (Cunningham and Wells, 1989; Bass et al., 1991; Bennett et al., 1991; Wertman et al., 1992). Alanine-scanning mutagenesis was first used by Cunningham and Wells (1989) to identify side chains on the human growth hormone that are involved in binding to the human growth hormone receptor. The majority of their mutants (81%) were expressed as stable proteins and of these 24% had significantly altered binding affinities for the receptor. Clustered charge-to-alanine mutagenesis has since been used to identify residues on the surface of proteins important for protein-protein interactions (Bass et al., 1991; Bennett et al., 1991; Wells et al., 1993), catalytic activities (Diamond and Kirkegaard, 1994), and to create a number of mutants having temperature sensitive (*ts*) phenotypes (Wertman et al., 1992; Hassett and Condit, 1994). We will present evidence that the charged residues from aa 114 to 129 of the NP protein are required for the nucleocapsid to function as a template in RNA replication *in vivo*. This defect is not due

to the lack of NP-P, NP-NP, or nucleocapsid-polymerase (P/L-Nuc) interactions in these mutants.

Results

Effect of Charge-to-Alanine Mutagenesis of the NP Protein on Sendai Virus RNA Replication *In Vitro*

We constructed six charge-to-alanine mutants in the charged region from aa 107 to 129 as described in Materials and Methods (Fig. 1). Within this charged region, aa 107 to 115 have been proposed as a putative RNA binding site (Morgan et al., 1984; Buchholz et al., 1993). The *in vitro* replication assay, described in Materials and Methods, was used to test the activity of the mutant NP proteins synthesized in cells cotransfected with the mutant NP plasmids (in place of the wt NP plasmid) and the P and L plasmids.

Initially, a single charge-to-alanine mutation (Glu to Ala, E107A) at aa 107 (NP107) was constructed. The mutant was expressed in VVT7-infected A549 cells that were transfected in duplicate with either the plasmid containing the gene for wt NP or NP107 as described in Materials and Methods. The cells were Tran^{35}S-labeled at 5.5 h p.t. for 30 min and extracts prepared immediately (pulse) or at 24 h p.t. following a chase in medium containing 10-fold excess methionine and cysteine (chase). The proteins were immunoprecipitated with an α-SV antibody and separated by SDS-PAGE as described in Materials and Methods. The wt and

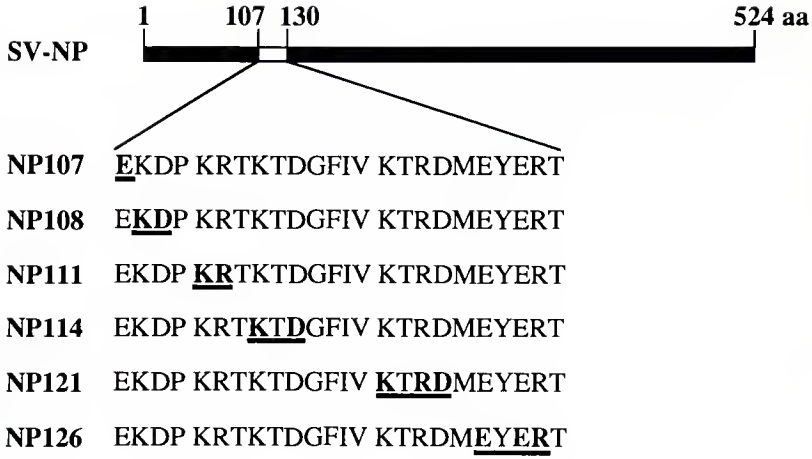


Figure 1. A schematic of the NP protein and the amino acid sequences (aa) from 107 to 130 of the charge-to-alanine NP mutants. Alanine was substituted for one, two, or three charged residues in the mutant NP proteins and these residues are highlighted by bold lettering and underlined.

the mutant NP proteins were found to be equally expressed in the pulse (Fig. 2B, lanes 3 and 5) and both proteins were stable to an overnight chase (Fig. 2B, lanes 4 and 6). The other bands in the gel are vaccinia virus proteins that were nonspecifically immunoprecipitated with the α-SV antibody as seen in the VVT7-infected, but not transfected cell extracts (Fig. 2B, lanes 1 and 2).

To test the biological activity of the mutant, extracts of cells expressing wt NP or NP107 with the P and L proteins were incubated with detergent disrupted SV DI-H and assayed for RNA genome replication *in vitro* as described in Materials and Methods. This assay measures one round of replication, i.e., the ability of the expressed NP protein to encapsidate the nascent RNA. The ^{32}P-labeled nuclease resistant product nucleocapsids were purified by banding on CsCl gradients, the RNA extracted, and analyzed by gel electrophoresis. Replication with the mutant protein, NP107, was somewhat better than replication with the wt NP protein (Fig. 2C, lanes 3 and 2, respectively). Replication of SV DI-H RNA was dependent on the expression of the SV NP, P, and L proteins since no activity was observed in an extract of VVT7-infected, but not transfected cells (Fig. 2C, lane 1). This mutant gave a phenotype indistinguishable from that of wt NP so this charged amino acid is not essential for activity.

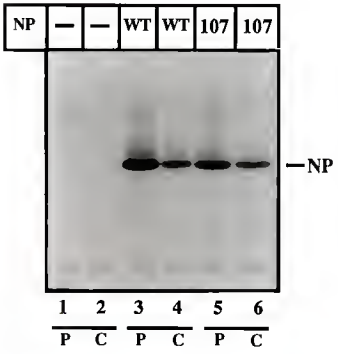
Based on these results we decided to change clustered groups of charged residues to alanine in the remaining five

Figure 2. Pulse-chase analysis and *in vitro* replication with the mutant NP107. A) Amino acid sequences (aa 107-130) of the wt NP and the mutant NP107 protein as described in Fig. 1. *In vitro* replication (%) with the mutant is shown relative to wt NP (100%). B) A549 cells (4.8×10^6) were infected with VVT7 and transfected in duplicate with no plasmids (-), the wt (WT) or mutant NP (107) (2 µg) plasmid, as indicated at the top. At 5.5 h p.t. the cells were labeled for 30 min with Tran^{35}S-label and extracts prepared immediately (pulse, P) or at 24 h p.t. following a chase (C). The proteins were immunoprecipitated with an α-SV antibody and analyzed by 9% SDS-PAGE. The position of the NP protein is indicated. C) A549 cells (4.8×10^6) were infected with VVT7 and transfected with no plasmids (-), or cotransfected with the P (5 µg) and L (0.5 µg) plasmids together with the wt or mutant NP (2 µg) plasmid, as indicated at the top. Cytoplasmic cell extracts were prepared at 18 h p.t. and incubated with detergent disrupted purified DI-H (dd DI-H) in the presence of [α-^{32}P]CTP. The nuclease resistant product nucleocapsids were banded on CsCl gradients and the purified RNAs were analyzed by gel electrophoresis as described in Materials and Methods. The position of the DI-H RNA is indicated and the amount of product DI-H RNA (as indicated in A) was quantitated on a phosphorimager.

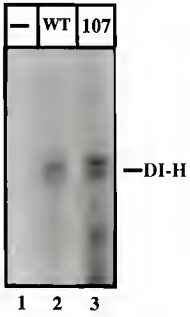
A

		% REPLICATION in vitro
WT NP	EKDP KRTKTDGFIV KTRDMEYERT	**100**
NP107	EKDP KRTKTDGFIV KTRDMEYERT	**141**

B



C



mutants (Fig. 1) with the hope that we would produce a stable protein with a mutant phenotype. Each of the mutant NP proteins, NP108, NP111, NP114, NP121, and NP126, were shown to be expressed as stable proteins by pulse-chase analysis as described in Materials and Methods (data not shown) and immunoblot analysis (Fig. 3D and E).

The mutant proteins were then tested for activity in SV DI-H RNA replication *in vitro* as described in Materials and Methods (Fig. 3). Replication of the detergent disrupted SV DI-H template with the wt NP protein was set at 100% (Figs. 3B and C, lanes 2 and 1, respectively). The level of replication with the mutants NP108, NP114, and NP121, was nearly equal or equivalent to wt NP replication at 80%, 104%, and 89%, respectively (Fig. 3B, lanes 3, 5, and 6). Replication with the mutants NP111 and NP126 was somewhat reduced, but was still significant with levels at 50% of wt NP (Fig. 3B, lane 4 and Fig. 3C, lane 2, respectively). The wt and mutant NP proteins were equally expressed in these extracts as shown by immunoblot analysis on a portion of the extracts (10%) with an α-SV antibody as described in Materials and Methods (Fig. 3D, lanes 2 to 6 and Fig. 3E, lanes 1 and 2). The P protein was also equally expressed, although the level of P protein detected by this antibody is less than that of the NP protein. These data show that the charged residues encompassing aa 107 to 129 in the NP protein can be changed in clusters to alanine without significantly

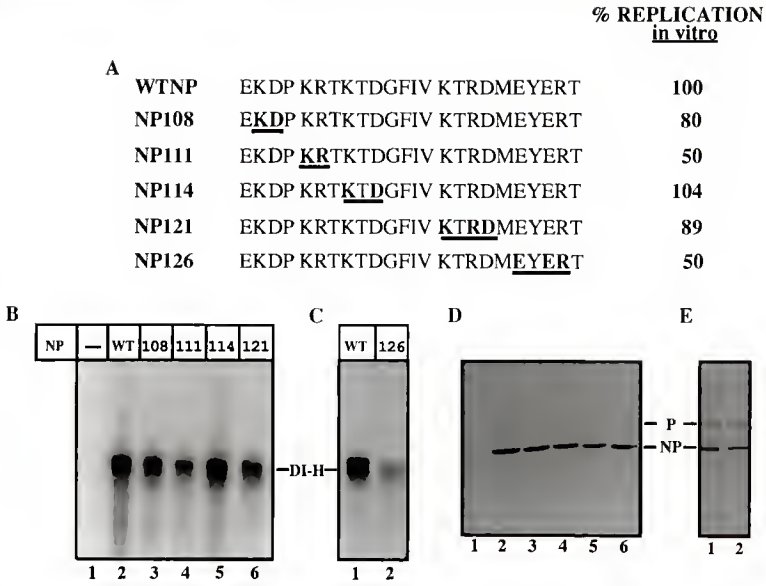


Figure 3. *In vitro* DI-H RNA synthesis with the charge-to-alanine mutant NP proteins. A) Amino acid sequence (aa 107-130) of the wt NP and the five mutant NP proteins (NP108, NP111, NP114, NP121, and NP126) and the level of replication (%) with these mutants as described in Fig. 2. B) and C) A549 cells were VVT7-infected and transfected with no plasmids (-), or cotransfected with the P and L plasmids together with the wt or mutant NP plasmid as indicated at the top and described in Fig. 2. Cytoplasmic cell extracts were prepared at 18 h p.t. and *in vitro* replication was assayed as described in Fig. 2. The position of the DI-H RNA is indicated and the amount of product DI-H RNA was quantitated on a phosphorimager. D) and E) Immunoblot analysis on samples (10%) of the cytoplasmic cell extracts used for *in vitro* replication with an α-SV antibody as described in Materials and Methods. The positions of the NP and P proteins are indicated. The sample numbers in B) and C) correspond to those in D) and E), respectively.

affecting (≤ 50%) the ability of the proteins to encapsidate nascent RNA *in vitro*.

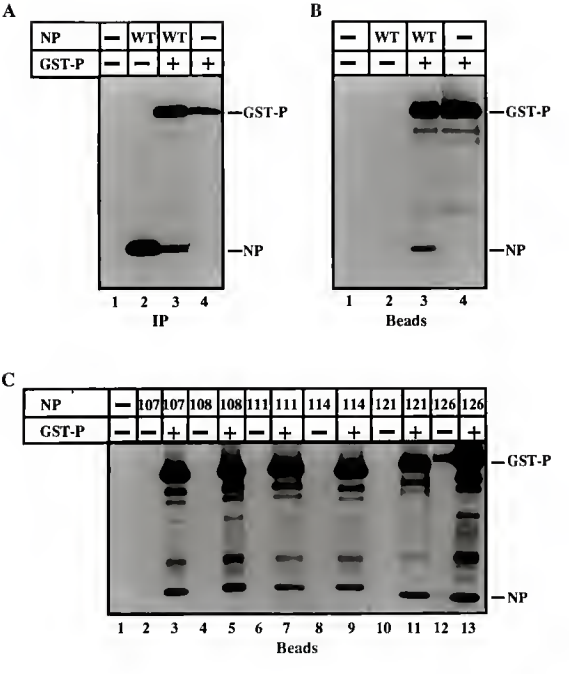
Protein-Protein Interactions

NP-P complex formation is conserved in the mutant NP proteins. One of the requirements for *in vitro* SV RNA replication is the formation of the encapsidation substrate, the NP-P complex (Horikami et al., 1992). Since each of the mutant proteins was active in replication *in vitro* to some degree, we expected that they were forming NP-P complexes, but this needed to be confirmed based on previous results with the measles virus nucleocapsid protein (MV N) (Chandrika et al., 1995). In this case, we showed that the MV N protein supported Sendai virus *in vitro* replication and yet this activity did not depend on complex formation between the MV N and SV P proteins. To measure NP-P complex formation we utilized a fusion protein containing the glutathione S-transferase protein fused to the N-terminus of the full-length Sendai virus P protein (GST-P). The wt or mutant NP proteins were expressed individually or together with the GST-P protein using the mammalian expression system in the presence of Tran^{35}S-label as described in Materials and Methods. Cell extracts were prepared at 18 h p.t. and identical aliquots were incubated with either glutathione Sepharose beads or an α-SV antibody as described in Materials and Methods. The bound and immunoprecipitated proteins were analyzed by SDS-PAGE.

Significant levels of the wt NP and GST-P proteins were expressed in the cell extracts as shown by immunoprecipitation with an α-SV antibody (Fig. 4A, lanes 2 through 4). The GST-P protein expressed alone bound to the beads (Fig. 4B, lane 4) as expected and the additional bands are apparently proteolysis products of the GST-P fusion protein, which still retain GST and, thus, bind to the beads. Proteins from VVT7-infected, but not transfected cell extracts did not bind to the beads (Fig. 4B, lane 1). The wt NP protein forms a complex with the GST-P protein as demonstrated by the NP protein cobinding to the beads when NP is coexpressed with GST-P (Fig. 4B, lane 3), but not when it is expressed alone (Fig. 4B, lane 2). The amount of the transfected pGEMSV-NP and pGST-P plasmids (2:1) was adjusted from the usual NP-P amounts (2:4) because the GST-P gene is overexpressed relative to the P gene in the mammalian expression system due to an internal ribosome binding site in the fusion protein mRNA. We showed that the NP protein does not form a complex with the GST protein alone (data not shown); therefore, the cobinding of NP with GST-P is specific for NP and P complex formation. Previously, NP-P complex formation had been demonstrated by coimmunoprecipitation and cosedimentation (Horikami et al., 1992).

Each of the mutant NP proteins NP107, NP108, NP111, NP114, NP121, and NP126, formed complexes with the P protein as well as evidenced by their cobinding with GST-P to the

Figure 4. Cobinding of the NP protein with the GST-P protein to glutathione Sepharose beads. A549 cells (4.8×10^6) were infected with VVT7 and transfected with no plasmids (-), the wt or mutant NP (2 µg) plasmid alone or together with the GST-P (1 µg) plasmid, or with the GST-P (1 µg) plasmid alone, as indicated. From 6-18 h p.t. the cells were labeled with Tran^{35}S-label as described in Materials and Methods. Cell extracts were prepared and samples were immunoprecipitated with an α-SV antibody and analyzed by 9% SDS-PAGE as described in Materials and Methods (A). Identical sample portions were incubated with glutathione Sepharose beads and the bound proteins were analyzed by 9% SDS-PAGE as described in Materials and Methods (B and C). The position of the NP and GST-P proteins are indicated.


A
NP
GST-P
WT
WT
GST-P
NP
1 2 3 4
IP
B
WT
WT
GST-P
NP
1 2 3 4
Beads
C
NP
GST-P
107 107 108 108 111 111 114 114 121 121 126 126
GST-P
NP
1 2 3 4 5 6 7 8 9 10 11 12 13
Beads

beads (Fig. 4C, lanes 3, 5, 7, 9, 11, and 13). In the absence of the GST-P protein none of the NP proteins bound to the beads (Fig. 4C, lanes 2, 4, 6, 8, 10, and 12). For simplicity only the bead binding data are shown, but all the proteins were synthesized to significant levels as detected by immunoprecipitation with an α-SV antibody (data not shown).

The mutant NP proteins can self-assemble and form nucleocapsidlike particles. In addition to the NP-P complex, an NP-NP interaction is also presumed to be required during genome RNA replication since RNA synthesis is coincident with cooperative encapsidation by the NP protein (Kolakofsky et al., 1991). Additional evidence for an NP-NP interaction was shown by self-assembly of the NP protein expressed alone into nucleocapsidlike particles, which were identical in morphology to authentic nucleocapsids from virus infected cells (Buchholz et al., 1993). Self-assembly of the NP protein occurred in the absence of any additional SV proteins or SV RNA. The density of the self-assembled wt NP protein was determined by banding the radiolabeled nucleocapsidlike particles synthesized *in vivo* on CsCl gradients as described in Materials and Methods. The majority of the self-assembled NP protein banded at the density of 1.30 gm/cc identical to authentic SV nucleocapsids (data not shown; see Fig. 14C).

The individual wt or mutant NP proteins were expressed using the mammalian expression system and banded on CsCl

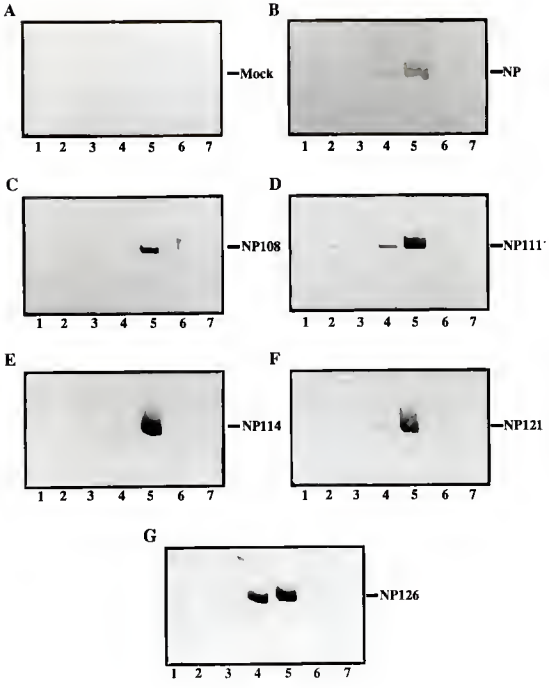
gradients as described in Materials and Methods. Fractions were collected from the top, separated by SDS-PAGE, and analyzed by immunoblotting with an α-SV antibody as described in Materials and Methods (Fig. 5). Consistent with previous data (Buchholz et al., 1993) the majority of the wt NP protein self-assembled into nucleocapsidlike particles as shown by its banding in fraction 5 at the 30-40% interface (Fig. 5B) like that of authentic nucleocapsids (Kolakofsky, 1976; Buchholz et al., 1993;). Cells VVT7-infected, but not transfected showed no viral protein (Fig. 5A) as expected.

Analysis of the self-assembly of the mutants NP108, NP111, NP114, NP121, and NP126 showed that the majority of each protein banded in fraction five identically to wt NP protein. These results show that all the mutant proteins self-assembled into nucleocapsidlike particles. In summary, these data show that substitution of alanine for the charged residues between aa 107 and 129 did not significantly disrupt DI-H RNA replication *in vitro* or NP-P or NP-NP protein complex formation

Charge-to-Alanine Mutagenesis of the NP Protein Disrupts *In Vivo* Replication

One limitation of the *in vitro* replication assay is that it does not measure if the genome RNA encapsidated by the mutant protein can then be used as a template for further rounds of replication (Curran et al., 1993). To measure this template function we have utilized a SV DI-H clone (pSPDI-H)

Figure 5. CsCl step gradient centrifugation of the self-assembled wt and mutant nucleocapsidlike particles. A549 cells (4.8×10^6) were VVT7-infected and transfected with no plasmids (mock), the wt (NP) or mutant NP (2 μg) plasmid, as indicated. At 20 h p.t. unlabeled cell extracts were prepared and analyzed by banding on separate CsCl step gradients as described in Materials and Methods. Fractions were collected from the top of the gradient and the pellet was resuspended in the last fraction (sedimentation is from left to right). Samples were analyzed by immunoblotting with an α-SV antibody as described in Materials and Methods. The positions of the wt or mutant NP proteins are indicated.


A
Mock
1 2 3 4 5 6 7
B
NP
1 2 3 4 5 6 7
C
NP108
1 2 3 4 5 6 7
D
NP111
1 2 3 4 5 6 7
E
NP114
1 2 3 4 5 6 7
F
NP121
1 2 3 4 5 6 7
G
NP126
1 2 3 4 5 6 7

that was constructed to contain the DI-H genomic sequence cloned downstream of the T7 promoter with the hepatitis delta virus ribozyme at the 3' end to generate authentic viral ends as described in Materials and Methods. In the mammalian expression system when the DI-H clone is cotransfected with the NP, P, and L plasmids into VVT7-infected A549 cells, the plus strand (+)DI-H RNA is transcribed by the T7 polymerase and encapsidated by the expressed NP protein. The (+)DI-H RNA-NP is then replicated by the expressed NP, P, and L proteins to amplify the template. Using this assay we can determine if the RNA encapsidated by the mutant NP proteins, where the mutant NP plasmid is substituted for the wt NP plasmid, can be used as a template for replication. Replication can be measured either by and assaying the amount of DI-H RNA produced by *in vitro* RNA synthesis or by northern analysis of the RNA extracted directly from the cells as described in Materials and Methods.

The cells were infected and transfected as described in Materials and Methods and after 18 h at 37°C lysolecithin cell extracts were prepared. Replication was detected first by direct labeling with [α-^{32}P]CTP at 30°C for 2 h and the replication products were purified and analyzed by gel electrophoresis as described in Materials and Methods. The DI-H RNA is not replicated in the absence of SV proteins (Fig. 6B, lane 1) as expected. Compared to replication with wt NP set at 100% (Fig. 6B, lane 2), the RNAs encapsidated by

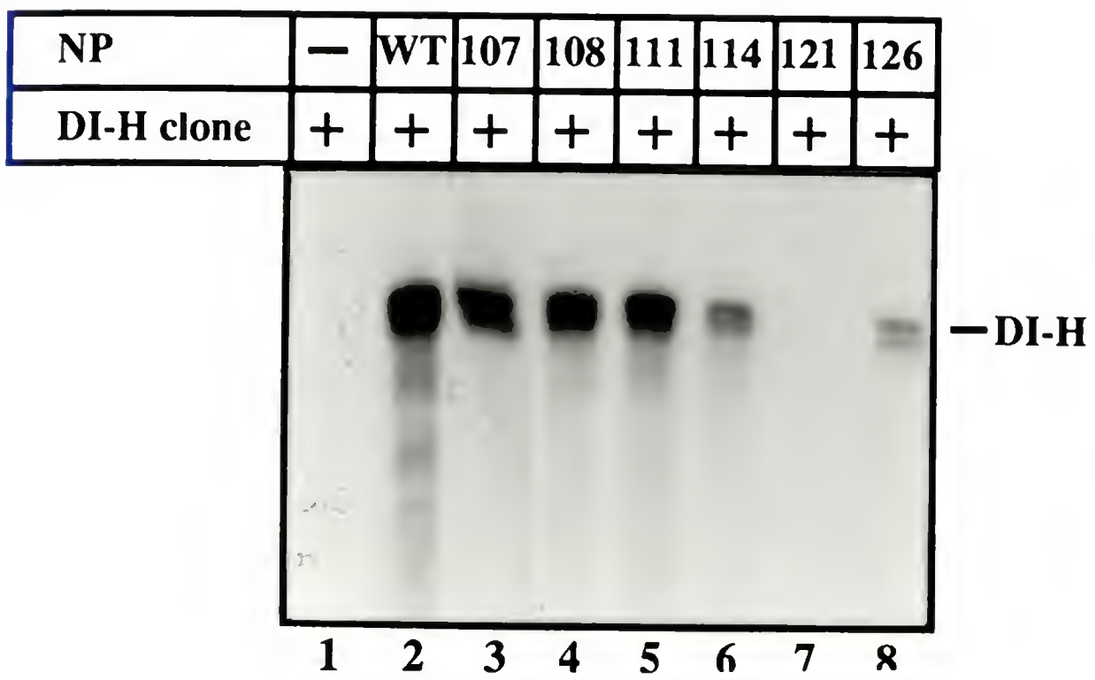
the mutant proteins NP107, NP108, and NP111, showed somewhat reduced, but still significant levels of RNA replication of 71%, 68%, and 69%, respectively (Fig. 6B, lanes 3, 4, and 5). RNAs encapsidated by the mutant proteins NP114 and NP126, however, were significantly impaired in template function (Fig. 6B, lanes 6 and 8; 28% and 18%, respectively) and the RNA encapsidated by NP121 was completely inactive (Fig. 6B, lane 7; 0%). Immunoblot analysis on samples of the cell extracts (10%) with an α-SV antibody demonstrated that the wt and mutant NP proteins were equally expressed (Fig. 6C, lanes 2 to 8) as described in Materials and Methods. These results indicate that while the mutants NP114, NP121, and NP126, are capable of encapsidating the nascent RNA during the first round of replication using DI-H encapsidated with wt NP as a template (Fig. 3), the product RNAs encapsidated by these mutant proteins do not function as templates for further rounds of replication.

Clustered charge-to-alanine mutations in a variety of proteins have been shown to yield a proportion of *ts* mutants (Wertman et al., 1992; Hassett and Condit, 1994; Diamond and Kirkegaard, 1994). We asked whether the NP mutants were *ts* and if we could rescue the template function of the mutants NP114, NP121, and NP126, by lowering the temperature of incubation. This was done by measuring the product nucleocapsid RNA synthesized *in vivo* by northern analysis and

A

		% REPLICATION
WTNP	EKDP KRTKTDGFIV KTRDMEYERT	**100**
NP107	**E**KDP KRTKTDGFIV KTRDMEYERT	**71**
NP108	E**KD**P KRTKTDGFIV KTRDMEYERT	**68**
NP111	EKDP **KR**TKTDGFIV KTRDMEYERT	**69**
NP114	EKDP KRT**KTD**GFIV KTRDMEYERT	**28**
NP121	EKDP KRTKTDGFIV **KTRD**MEYERT	**0**
NP126	EKDP KRTKTDGFIV KTRDM**EYER**T	**18**

B



C

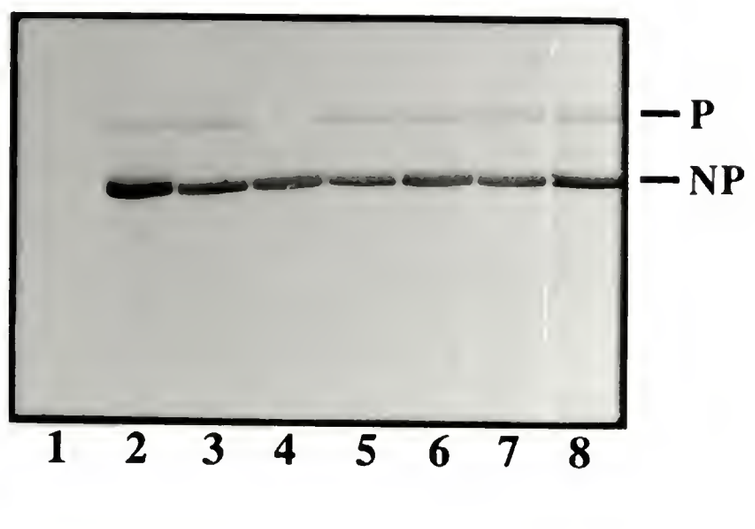


Figure 6. Template function of the (+)DI-H RNA-NPs assembled *in vivo* with wt or mutant NP proteins. A) Amino acid sequence (aa 107-130) of the wt NP and the six NP mutant proteins and the level of replication *in vitro* (%) with each mutant template as described in Fig. 1. B) VVT7-infected A549 cells were transfected with the DI-H (2.5 μg) plasmid, or cotransfected with the DI-H plasmid (2.5 μg), the P (5 μg) and L (0.5 μg) plasmids together with the wt or the mutant NP (2 μg) plasmid, as indicated. Cytoplasmic cell extracts were prepared at 18 h p.t. and *in vitro* replication was assayed as described in Fig. 2. The position of the DI-H RNA is indicated and the amount of product DI-H RNA was quantitated on a phosphorimager. C) Immunoblot analysis on a portion (10%) of the cytoplasmic cell extracts as described in Fig. 3.

does not require the temperature shift between expression and the assay that was necessitated by the experiments above.

A549 cells were infected with VVT7 at 37°C, transfected in duplicate and then incubated either at 32°C for 36 h or at 37°C for 22 h. Nuclease resistant RNA was banded on CsCl gradients and the RNA extracted as described in Materials and Methods. The purified RNA was separated by gel electrophoresis and transferred to nitrocellulose. An unlabeled *in vitro* DI-H reaction was included to provide a marker replication product (Fig. 7B, lane 10) and DI-H RNA was detected by northern blotting with a randomly labeled DI-H probe. There was no replication in VVT7-infected cells transfected with only the DI-H plasmid, showing that replication was dependent on expression of the NP, P, and L proteins (Fig. 7B, lane 1). The nucleocapsid RNA produced with wt NP at 32°C (Fig. 7B, lane 2; 100%) was reproducibly greater than at 37°C (Fig. 7B, lane 3; 29%). Replication with NP114, however, was greater at 37°C (Fig. 7B, lane 5; 47%) than at 32°C (Fig. 7B, lanes 4; 17%), suggesting that this mutant has a cold sensitive (*cs*) phenotype. The template function of NP126 also appeared to be cold sensitive (Fig. 7B, lanes 8 and 9; 5 and 20%), but at these low levels of replication this remains speculative. The mutant NP121 was completely inactive in replication at both temperatures (Fig. 7B, lanes 6 and 7; 0%), and is evidence that the RNA encapsidated by the mutant NP121 is not used as a template

A

		% REPLICATION in vivo 32°C	37°C
WTNP	EKDP KRTKTDGFIV KTRDMEYERT	100	29
NP114	EKDP KRTKTDGFIV KTRDMEYERT	17	47
NP121	EKDP KRTKTDGFIV KTRDMEYERT	0	0
NP126	EKDP KRTKTDGFIV KTRDMEYERT	5	20

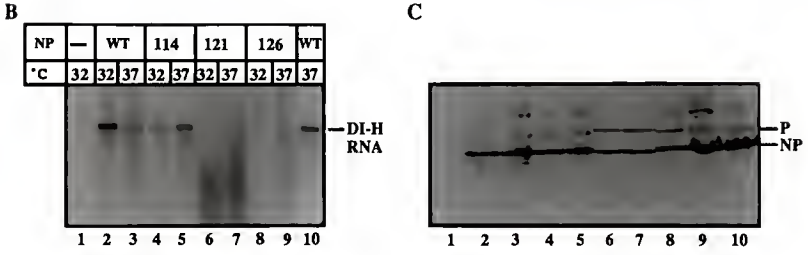


Figure 7. Northern analysis of DI-H RNA replication *in vivo*. A) Amino acid sequence (aa 107-130) of the wt and the mutant NP proteins as described in Fig. 1. *In vivo* replication (%) with the wt and mutant NP proteins at 32°C and 37°C are shown relative to wt NP at 32°C (100%). B) A549 cells (1.8 x 10^7) were VVT7-infected and transfected as described in Fig. 6, with following amount of plasmids: 7.5 µg DI-H plasmid, 15 µg P plasmid, 1.5 µg L plasmid, and 6 µg wt or mutant NP plasmid, and incubated at either 32°C or 37°C, as indicated. Cytoplasmic cell extracts were prepared at 36 h or 22 h (32°C and 37°C, respectively) and analyzed by northern blotting as described in Materials and Methods. The product DI-H RNA was identified with a randomly labeled ^{32}P-DI-H probe. An unlabeled *in vitro* DI-H reaction was included to provide a marker replication product (lane 10). The position of the DI-H RNA is indicated. C) Immunoblot analysis on a portion (10%) of the cell extracts using enhanced chemiluminescence with an α-SV antibody as described in Materials and Methods. The positions of the NP and P proteins are indicated.

for RNA replication under any of the conditions tested. Immunoblot analysis with an α-SV antibody showed that the wt and mutant NP proteins and the P protein were equally expressed in these cell extracts (Fig. 7C, lanes 2 through 10). The other bands in the blot represent vaccinia proteins nonspecifically staining in this particular blot.

Binding of the SV Polymerase to the Wild-Type and Mutant Self-Assembled Nucleocapsidlike Particles

One requirement for viral RNA replication is the recognition of and binding of the viral polymerase through the P moiety to the template (P/L-Nuc protein complex). To determine if the template defects identified in the *in vivo* replication assays were due the inability of the polymerase to bind to the mutant nucleocapsids, we tested binding with self-assembled nucleocapsidlike particles as described previously (Horikami and Moyer, 1995). The wt and mutant NP proteins representing each defective phenotype seen in replication *in vivo* (significant, reduced, and no activity) were self-assembled by the expression of each protein in VVT7-infected and transfected A549 cells in the presence of Tran^{35}S-label. The radiolabeled nucleocapsidlike particles were purified by sedimentation through glycerol and resuspended as described in Materials and Methods. Equal aliquots of the extracts expressing the radiolabeled viral polymerase proteins were incubated with the nucleocapsidlike particles. As a positive control, purified polymerase-free

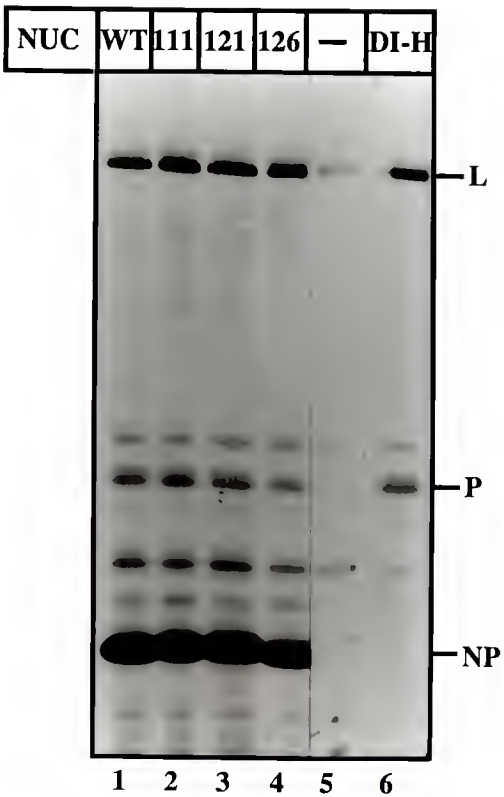
SV DI-H (RNA-NP template) was also incubated with the viral polymerase extract. The reactions were then sedimented through glycerol, the pellets collected, and immunoprecipitated with the α-SV and α-L antibodies to monitor the proteins associated with the nucleocapsids.

We showed that the P and L proteins copellet (i.e., bind) in the presence of both the SV RNA-NP template and the wt nucleocapsidlike particles (Fig. 8, lanes 6 and 1, respectively), but only a small amount of the polymerase proteins pelleted in the absence of nucleocapsid template (Fig. 8, lane 5), in agreement with previous data. The polymerase proteins bound to the three mutant self-assembled nucleocapsidlike particles (Fig. 8, lanes 2 to 4) at levels equal to wt nucleocapsids as indicated by their copelleting. The additional bands on the gel are vaccinia virus proteins that pelleted and were nonspecifically immunoprecipitated in this assay. Thus, the mutant nucleocapsidlike particles still have the binding sites for the viral polymerase. These results show that the template defect in the mutant nucleocapsids manifested during replication *in vivo* is not due to lack of binding of the viral RNA polymerase to the templates.

Discussion

A summary of the properties of the charge-to-alanine NP mutants from aa 107-129 is presented in Fig. 9. This approach identified a region of the NP protein from aa 114

Figure 8. Binding of the viral polymerase to the wt and mutant self-assembled nucleocapsidlike particles. As a source of nucleocapsidlike particles, VVT7-infected A549 cells (4.8×10^6) were transfected with no plasmids (-), or the wt or the mutant NP plasmid (2 μg), as indicated. As a source of the viral polymerase, VVT7-infected A549 cells were cotransfected with the P (5 μg) and L (5 μg) plasmids. At 6 h p.t. the cells were Tran^{35}S-labeled for 3 h (nucleocapsidlike particles) or 1 h (viral polymerase) and cell extracts were prepared as described in Materials and Methods. The mock cell extract (-) and the nucleocapsidlike particles were purified by pelleting separately through glycerol. An equal aliquot of the viral polymerase extract was incubated separately with the purified mock extract (lane 5), each of the purified nucleocapsidlike particles (lanes 1 to 4), and purified polymerase-free SV DI-H (RNA-NP, lane 6). The samples were pelleted through glycerol as described in Materials and Methods. The pellets were resuspended and the bound proteins were immunoprecipitated with the α-SV and α-L antibodies and analyzed by 7.5% SDS-PAGE. The positions of the NP, P, and L proteins are indicated.


NUC
WT
111
121
126
—
DI-H
L
P
NP
1
2
3
4
5
6

and 129 that was required for the nucleocapsid to function as a template, but was not required for RNA encapsidation. It was surprising that changing a total of 13 charged aa to alanine in these six mutant proteins had little effect on replication *in vitro*, i.e., encapsidation (Fig. 9). Previous analysis of a set of 22 NP deletion mutants by Curran et al. (1993) found that the entire region from aa 1-400 was required for *in vitro* replication. These apparently conflicting results are likely due to the different effects on protein structure by the alanine substitutions versus the deletions.

Alanine substitutions generally have minimal effects on protein folding or conformation (Cunningham and Wells, 1989; Bennett et al., 1991; Bass et al., 1991; Wertman et al., 1992). In contrast, deletions are more likely to affect protein folding leading to multiple alterations in protein domains downstream of the deletion. Furthermore, misfolded proteins are subject to proteolysis *in vivo* and this may be the reason that synthesis of two mutant NP proteins containing deletions between aa 107-130 or 167-187 was not sufficient for biochemical analysis (Buchholz et al., 1993). Additional evidence that the mutant proteins encompassing aa 107-129 are folded correctly was that each of the six mutants formed both NP-P and NP-NP complexes (Fig. 9) (Predki et al.,1995).

	COMPLEX FORMATION			VIRAL RNA REPLICATION (%)		
				In Vitro	In Vivo	
NP PROTEIN	NP:P	NP:NP	P/L:NUC	α^{32}P Label	α^{32}P Label	Northern
NP	+	+	+	100	100	+
NP107	+	ND	ND	141	71	ND
NP108	+	+	ND	80	68	ND
NP111	+	+	+	50	69	ND
NP114	+	+	ND	104	28	CS
NP121	+	+	+	89	0	0
NP126	+	+	+	50	18	CS

Figure 9. A summary of the protein-protein interactions and *in vitro* DI-H RNA replication data of the wt and mutant NP proteins. The data for the NP:P, NP:NP, and P/L:NUC interactions are from Figs. 4, 5, and 8 and the data for (%) RNA replication are from Figs. 2, 3, 6, and 7.

One possibility for the observation that the nucleocapsids formed with the mutants NP114, NP121, and NP126, did not function as templates for replication (Fig. 9) could be that the RNA polymerase could not bind. Binding of the polymerase to the template is mediated through the P protein (Portner and Murti, 1986; Portner et al., 1988; Morgan, 1991; Horikami and Moyer, 1995). We showed that the viral polymerase, i.e., the P and L proteins, bound to the mutant self-assembled nucleocapsids at wild-type levels (Fig. 9) suggesting that the template defect is not due to lack of polymerase binding to the template. Clearly, polymerase binding does not require these charged residues of NP. Interestingly, it has been shown that any NP mutant containing deletions within the C-terminal tail (aa 400-524) had phenotypes similar to those exhibited by NP114, NP121, and NP126 (Curran et al., 1993). That is, deletions of aa 400-415, 414-439, 426-497, and 456-524 formed NP-NP complexes, encapsidated RNA *in vitro*, but were inactive in replication *in vivo*. More recently, a P (polymerase) binding site has been mapped within the C-terminal tail of the self-assembled NP protein between aa 440-524 (Buchholz et al., 1994). Therefore, the mutants containing deletions of aa 426-497 or 456-524 could not replicate *in vivo* because the polymerase could not bind. However, the mutants with deletions of aa 400-415 or 414-439, like the mutants NP114, NP121, and NP126, could not replicate either, yet bound the P

protein. In combination these data suggest that at least two sites are required for template function not related to polymerase binding. One is located in the C-terminal aa 400-439 and the other from aa 114-129 and that these two form a single domain in the mature protein. Alternatively, the evidence does not preclude the possibility that each of these sites act as a separate domain with unique functions.

Several models can be envisioned for the role of aa 114-129 and aa 400-439 in template function, depending on whether these sites form one domain or two, and if the bases of the RNA are exposed or buried in the nucleocapsid. The first three models are based on the hypothesis that the bases are covered by the NP protein, and that aa 114-129 and 400-439 form a single domain in the NP protein. In the first model, this domain would be required for the temporary displacement of NP to allow the viral polymerase access to the bases during RNA synthesis (Curran et al., 1993). As described previously, the temporary displacement of NP does not change the nuclease-resistant character of the template; therefore, the displacement of NP must be minimal and/or something else must take the place of the displaced NP (Curran et al., 1993). To accomplish this the NP protein may be displaced by its binding to the P subunit of the RNA polymerase, via aa 114-129 and aa 400-439, and the L subunit of the polymerase filling the gap (Curran et al., 1993). Following replication, the displaced NP protein would be returned. In

VSV, it has been proposed that the NS (P protein) protein may mimic the template RNA and act as a binding site for the N protein (Hudson et al., 1986).

In models two and three, the mutations have resulted in an increased affinity for either the RNA (NP-RNA interaction) or the adjacent NP proteins (NP-NP interaction), respectively. The increased affinity would prevent the temporary displacement of the mutant NP protein from the RNA or adjacent mutant NP proteins. This increased affinity would not affect *in vitro* replication since the RNA template is encapsidated by wt NP protein. A fourth model would propose that the aa 114-129 and 400-439 represent individual domains responsible for the separate functions described above or some other unidentified function of the NP protein.

When RNA encapsidation and amplification were all assayed at 37°C (northern) as compared to encapsidation and amplification at 37°C followed by an *in vitro* assay at 30°C (direct labeling), a degree of template function was restored to the mutant nucleocapsid containing NP114 (Figs. 7 and 9). Comparison of the two assays (direct labeling and northern blot) for template amplification showed that for NP114 shifting the temperature from 37°C to 30°C inhibited template function of the nucleocapsids, suggesting that this mutant has a cold sensitive (*cs*) phenotype. Both *ts* and *cs* mutants have been described previously in alanine-scanning mutants (Wertman et al., 1992; Hasset and Condit, 1994). In

conclusion, this approach has allowed us to create six stable mutant NP proteins and has identified a second region (aa 114-129) of the NP protein, in addition to the previously identified C-terminal 400-439 aa, required for template function of the nucleocapsid. Additional experiments will need to be developed that will determine if these two sites form one domain or fulfill separate functions in the nucleocapsid template.

CHAPTER 4
IDENTIFICATION OF A PUTATIVE RNA BINDING SITE

Introduction

The single-stranded negative sense RNA genome of Sendai virus is encapsidated by the NP protein forming an RNase resistant nucleocapsid and genome replication is coupled to its encapsidation with NP. The substrate for encapsidation *in vitro* is the NP-P complex (Horikami et al., 1992) and it is believed that the P protein is released once the NP protein binds to the nascent RNA (Howard and Wertz, 1989; Curran et al., 1995). This model predicts both a specific encapsidation signal in the SV RNA, which could be either an RNA sequence or RNA structure, and a specific RNA binding domain in the NP protein. For VSV, it has been shown that the binding affinity of the nucleocapsid protein for leader RNA is approximately 10 times greater than nonspecific RNA (Blumberg et al., 1983;), and the encapsidation signal is in the 3'-terminal 15-17 nt of the leader RNA (Moyer et al., 1991; Smallwood and Moyer, 1993). By analyzing mutant SV NP proteins for function in replication *in vitro* and for protein-protein interactions, we have identified specific NP amino acids required for the NP-NP interaction and a putative NP-RNA interaction.

Results

Construction of Mutant NP Genes

Based on the amino acid alignments of thirteen paramyxovirus NP proteins (Fig. 17) (Miyahara et al., 1992) we selected the region between aa 362 and 375 (Fig. 10) of the NP protein for site-directed alanine-scanning mutagenesis (Cunningham and Wells, 1989). This region was selected because it is adjacent to a conserved sequence (aa 257-358) that has been identified in thirteen different paramyxoviruses (Miyahara et al., 1992). The region from aa 362-375 also contains conserved hydrophobic residues (aa 362 to 369) and two conserved charged residues (aa 370 and 371) (Fig. 17). Charged residues found in the primary sequence of the protein are likely to be located on the surface of the protein suggesting that they could be interacting with other molecules or proteins (Cunningham and Wells, 1989; Bass et al., 1991). We propose that this region in the NP protein is conserved because it is important for some aspect of the NP protein structure and/or function. To control for bias in selecting only conserved residues we included two unconserved charged residues (aa 373 and 375) for mutagenesis.

To decrease the chance of disrupting the secondary structure of the protein, the targeted aa were changed to alanine. Alanine is found in both exposed and interior positions and it is less apt than other amino acid substitutions, insertions, or deletions to alter protein conformation (Cunningham and Wells, 1989; Bass et al., 1991;

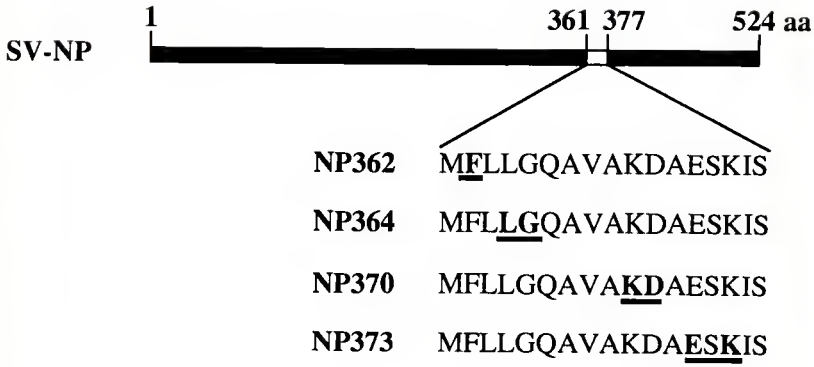


Figure 10. A schematic of the NP protein and the amino acid sequences (aa) from 361 to 377 of the alanine-scanning NP mutants. Alanine was substituted for one or two residues in the mutant NP proteins and these residues are highlighted by bold lettering and underlined.

Bennett et al., 1991; Wertman et al., 1992). For these reasons any recovered phenotype is likely due to removal of the specific side chains of the targeted amino acid.

To determine if the residues located from aa 362-375 are important for viral RNA synthesis we created four NP mutants, NP362, NP364, NP370, and NP373 (Fig. 10). Site-directed mutagenesis was used to substitute alanine for the indicated amino acids (Fig. 10).

Effect of Charge-to-Alanine Mutagenesis on Protein Stability

Protein expression and stability of each of the NP mutants was determined using the mammalian expression system as described in Materials and Methods. Infected and transfected A549 cells were radiolabeled for 30 min at 5.5 h p.t. and extracts prepared immediately (pulse) or following a 16 h chase in 10-fold excess methionine and cysteine (chase). The proteins were immunoprecipitated using an α-SV antibody and separated by SDS-PAGE as described in Materials and Methods. The wt NP protein was synthesized in the pulse and was stable to the chase (Fig. 11, lanes 3 and 4, respectively), as were the mutants NP362, NP370, and NP373 (Fig. 11, lanes 5, 6, 9, 10, 11, and 12, respectively).

The mutant NP364 protein was synthesized in the pulse, but it was not stable to the chase (Fig. 11, lanes 7 and 8, respectively). In this experiment, the level of synthesis of the mutant proteins, NP362 and NP364, was less than the wt NP

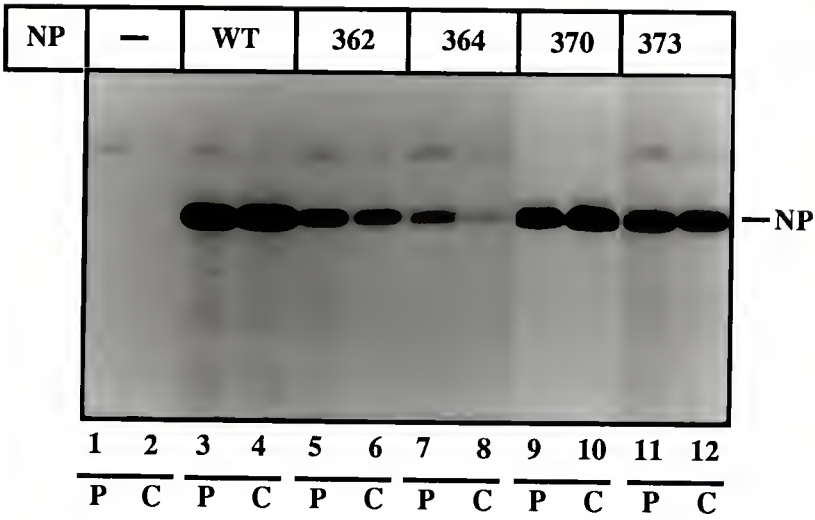


Figure 11. Pulse-chase analysis of the alanine-scanning mutants described in Fig. 10. A549 cells (4.8 x 10^6) were VVT7-infected and transfected in duplicate with no plasmids (-), the wt (WT) or mutant NP (2 μg) plasmid, as indicated at the top. At 6 h p.t. the cells were labeled for 30 min in methionine- and cysteine-free medium with Tran^{35}S-label and extracts prepared immediately (pulse, P) or at 16 h p.t. following a chase (C) as described in Materials and Methods. The proteins were immunoprecipitated with an α-SV antibody and analyzed by 9% SDS-PAGE. The position of the NP protein is indicated.

protein in the pulse, but this was determined to be due to a lesser amount of each plasmid transfected (see Figs. 13, 15E, and 15F). In a time course experiment it was determined that the NP364 protein was stable only for about 1.5 h following the pulse (data not shown). The lack of protein stability in the mutant NP364 has limited the number of assays that can be used to characterize this mutant and any subsequent experiments used extracts harvested during stable protein expression. These results suggested that substitution of the hydrophobic Leu and Gly side chains (aa 364 and 365, respectively; NP364) were destabilizing and led to proteolysis *in vivo* (Cunningham and Wells, 1989; Bass et al., 1991). While it is less likely that alanine substitutions will result in unstable mutant proteins (Cunningham and Wells, 1989; Bass et al., 1991), it has been reported that mutants containing multiple alanine substitutions were not expressed at levels sufficient for analysis (Diamond and Kirkegaard, 1994; Bass et al., 1991).

Two Charge-to-Alanine NP Mutants are Inactive in RNA Replication *In Vitro*

The mutant NP proteins NP362, NP370, and NP373 were assayed in duplicate for activity in DI-H genome replication *in vitro*. As described in Materials and Methods, cytoplasmic cell extracts were prepared at 18 h p.t., consequently the mutant NP364 was not included in this assay. Purified dd DI-H was mixed with the cytoplasmic cell extracts and incubated in the presence of [α-^{32}P]CTP at 30°C for 2 h. The

replication products were micrococcal nuclease treated and purified as described in Material and Methods.

In vitro DI-H RNA replication occurred with the wt NP protein and is arbitrarily set at 100% (Fig. 12B, lane 2), while the DI-H template was not replicated in the absence of Sendai virus proteins (Fig. 12B, lane 1). The mutants NP362 and NP370 were completely inactive in RNA replication *in vitro* (Fig. 12B, lanes 3 to 6). The level of replication with NP373 was two-fold greater than with wt NP protein (Fig. 12B, lanes 7 and 8). A portion (10%) of these cell extracts was used for immunoblot analysis with α-SV antibody as described in Materials and Methods. The wt and mutant NP proteins, as well as the P protein, were shown to be equally expressed (Fig. 12C, lanes 2 to 8), so protein was not limiting in the inactive NP mutants.

Substitution of alanine for the conserved Phe, Lys, and Asp at aa 362 (NP362); 370 and 371 (NP370); respectively, resulted in mutant NP proteins that were completely inactive in RNA replication *in vitro*. As discussed previously in the *in vitro* RNA replication assay, one round of replication is completed using a wt template; therefore, we are measuring the ability of the expressed NP protein to encapsidate the nascent RNA. The identified interactions that are required for *in vitro* RNA replication are the NP-P (encapsidation substrate), NP-NP, and NP-RNA. Disruption of any one of these interactions could be responsible for a negative result

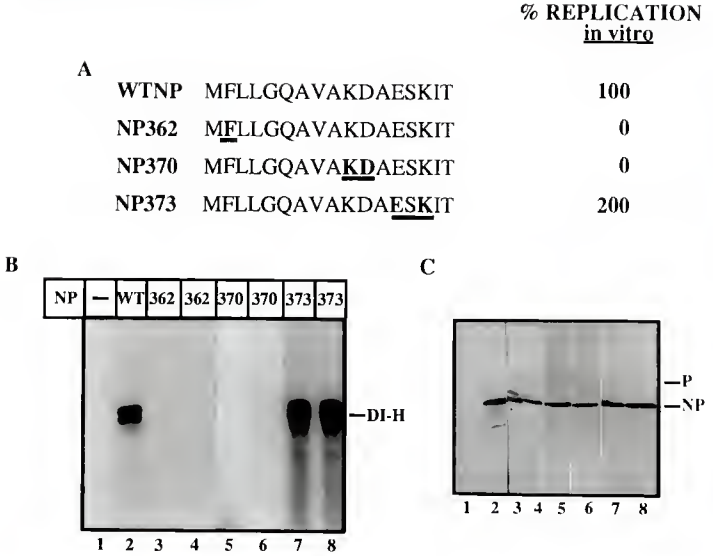


Figure 12. *In vitro* DI-H RNA replication with the mutant NP362, NP370, and NP373 proteins. A) Partial amino acid sequences (aa 361-377) of the wt NP and the mutant NP proteins as described in Fig. 10. *In vitro* replication (%) with the mutant is shown relative to wt NP (100%). B). A549 cells were VVT7-infected and transfected with no plasmids (-) or cotransfected with the P and L plasmids together with the wt or mutant NP plasmid, as described in Fig. 2. Cytoplasmic cell extracts were prepared at 18 h p.t. and *in vitro* replication was assayed as described in Fig. 2. The position of the DI-H RNA is indicated and the amount of product DI-H RNA (as indicated in A) was quantitated on a phosphorimager. C) Immunoblot analysis on samples (10%) of the cytoplasmic cell extracts as described in Fig. 3. The positions of the NP and P proteins are indicated. The sample numbers in C) correspond to those in B).

in RNA replication. Therefore, the individual protein-protein interactions for the mutants were tested.

NP-P Complex Formation is Preserved in the Charge-to-alanine Mutants

The GST-P fusion protein was used to assay whether the mutant NP proteins, NP362, NP370, and NP373, could form the NP-P protein complex. The mutant NP proteins were expressed alone or together with GST-P in A549 cells and the cells were Tran^{35}S-labeled from 5.5 to 17.5 h p.t. as described in Materials and Methods. The formation of an NP-P complex can be measured by cobinding of the NP protein with the GST-P protein to the glutathione Sepharose beads, as demonstrated earlier (Fig. 4). A portion of the cell extract was used for bead binding and an identical portion was used for immunoprecipitation as described in Materials and Methods. All of the mutant NP and GST-P proteins in the cell extracts were expressed to significant levels as detected by immunoprecipitation with α-SV antibody (data not shown).

Each of the mutant NP proteins cobound to the beads in the presence of GST-P (Fig. 13, lanes 2, 4, and 6), but not when expressed alone (Fig. 13, lanes 1, 3, and 5) and this was evidence that each of the mutant NP proteins formed a complex with the P protein. As a positive control for bead binding, the GST-P protein expressed singularly bound to the beads (Fig. 13, lane 7) and the additional bands in the gel are apparently proteolysis products of the GST-P protein. This is the first evidence suggesting that the domains in the

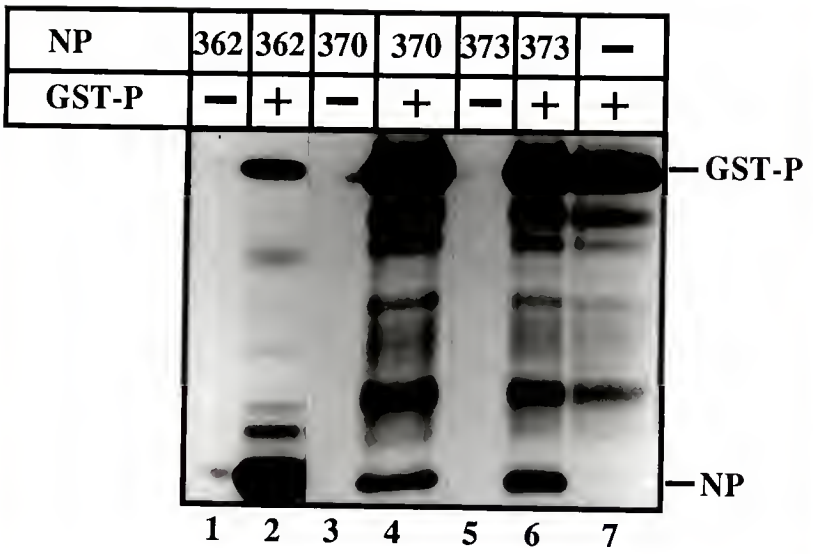


Figure 13. Cobinding of the mutant NP proteins with the GST-P protein to glutathione Sepharose beads. A549 cells were VVT7-infected and transfected with no plasmids (-), the mutant NP (2 µg) plasmid alone or together with the GST-P (1 µg) plasmid, or with the GST-P (1 µg) plasmid alone, as indicated. The cells were radiolabeled for 12 h and cell extracts prepared and analyzed by binding to glutathione Sepharose beads as described in Fig. 4. The position of the NP and GST-P proteins are indicated.

first 400 aa of the NP protein required for complex formation during *in vitro* replication can be separated phenotypically (Homann et al., 1991; Curran et al., 1993). That is, both the NP362 and NP370 mutant proteins formed NP-P complexes, yet both proteins were inactive in RNA replication *in vitro*.

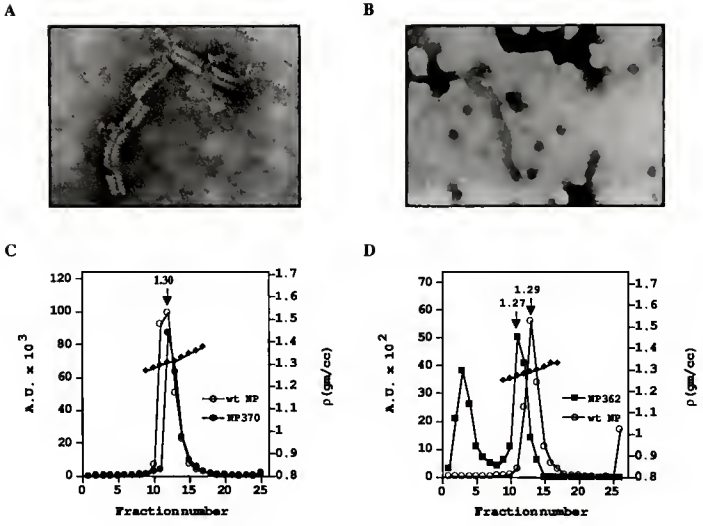
Self-Assembled Wild-Type and Mutant Nucleocapsidlike Particles Contain RNA

Initially, NP-NP complex formation, that is self-assembly, was assayed by examining purified cell extracts containing the wt NP or mutant NP370 protein by electron microscopy. The wt NP and mutant NP370 proteins were each expressed in A549 cells and the self-assembled nucleocapsidlike particles were purified by pelleting through 30% (v/v) glycerol (5 ml) as described in Materials and Methods. The pellets were resuspended in ET buffer, negatively stained using 2% uranyl acetate, and examined by electron microscopy. The mutant NP370 protein was shown to self-assemble into nucleocapsidlike particles that were identical in appearance to self-assembled wt NP nucleocapsidlike particles (Fig. 14B and A, respectively). The wt NP and mutant NP370 nucleocapsidlike particles were identical in morphology to nucleocapsids found in wt SV infected cell extracts (data not shown) and these results agree with previous reports on self-assembly of the SV NP protein (Buchholz et al., 1993) and the MV N protein (Spehner et al., 1991).

One property of the self-assembled NP370 protein that is not answered by the electron microscopy data is whether the mutant nucleocapsidlike particles contained RNA. Previous reports on self-assembled SV NP in mammalian cells showed that the self-assembled nucleocapsidlike particles contained RNA (Buchholz et al., 1993), and it was suggested that the MV N protein self-assembled in mammalian cells was also associated with RNA (Spehner et al., 1991). In contrast, self-assembled MV N nucleocapsidlike particles synthesized in insect cells did not appear to contain RNA as determined by their lighter buoyant density (Fooks et al., 1993). To determine whether the mutant NP370 nucleocapsidlike particle contained RNA, the wt and mutant proteins were expressed in A549 cells and the cells were radiolabeled at 5.5 h p.t. for 14 h prior to preparing cell extracts as described in Materials and Methods.

The radiolabeled proteins were analyzed by banding on separate linear CsCl gradients. Individual fractions were collected from the top of the gradient and the density of each fraction was determined using a refractometer. The protein in each fraction was immunoprecipitated with an α-SV antibody, separated by SDS-PAGE, and quantitated on the phosphorimager as described in Materials and Methods. The quantity of protein in each fraction and the density of each fraction are plotted in Fig. 14C. Both self-assembled wt NP and the mutant NP370 nucleocapsidlike particles had identical

Figure 14. Electron microscopy of the wt NP and NP370 proteins and CsCl gradient analysis of the wt NP, NP370, and NP362 proteins. A549 cells (4.8×10^6) were VVT7-infected and transfected with the wt or mutant NP (2 μg) plasmid and unlabeled cell extracts prepared 20 h p.t. (A and B), or labeled with Tran^{35}S-label from 5.5-19.5 h p.t.(C and D) as described in Materials and Methods. A) and B) The unlabeled cell extracts expressing either the wt NP or NP370 protein, respectively, were purified by pelleting through glycerol as described in Materials and Methods. The pellets were resuspended in ET buffer and negatively stained using 2% uranyl acetate and examined by electron microscopy. The magnification for A (wt NP) is twice that in B (NP370). C) Radiolabeled wt NP or NP370 proteins were analyzed by sedimentation on separate CsCl gradients, 0.5 ml fractions were collected, samples were immunoprecipitated with an α-SV antibody, and analyzed by 9% SDS-PAGE as described in Materials and Methods. Sedimentation is from left to right. The amount of label in the wt NP (o) or NP370 (●) protein in each fraction was quantitated on the phosphorimager and presented graphically. The density (♦) of each fraction was determined and the density of the peak fraction is indicated. D) As described in C, except the wt NP (o) and the NP362 (■) proteins were analyzed on parallel gradients.


A
B
C
D
1.30
wt MP
MP370
A.U. x 10^3
ρ (gm/cc)
Fraction number
1.29
1.27
MP362
wt MP
A.U. x 10^2
Fraction number

densities of 1.30 gm/cc (Fig. 14C), which was identical to the density of authentic SV nucleocapsids (Chandrika et al., 1995). It is noted that SV nucleocapsids have been reported to band at densities ranging from 1.29 to 1.33 gm/cc (Carlsen et al., 1985; Chandrika et al., 1995, and Buchholz et al., 1993), and the difference in the densities probably reflects the unavoidable variations associated with gradient preparation and density measurement. Nonetheless, these data show that the wt NP and the mutant NP370 nucleocapsidlike particles banded at the density of NP-RNA particles and this is evidence that the nucleocapsidlike particles contain RNA. These results agree with those reported by Buchholz et al. (1993) on SV NP protein self-assembly. Additionally, the viral polymerase bound to the self-assembled NP370 particles analogous to the wt NP protein (Figure 8), showing that the P/L binding site was intact (data not shown).

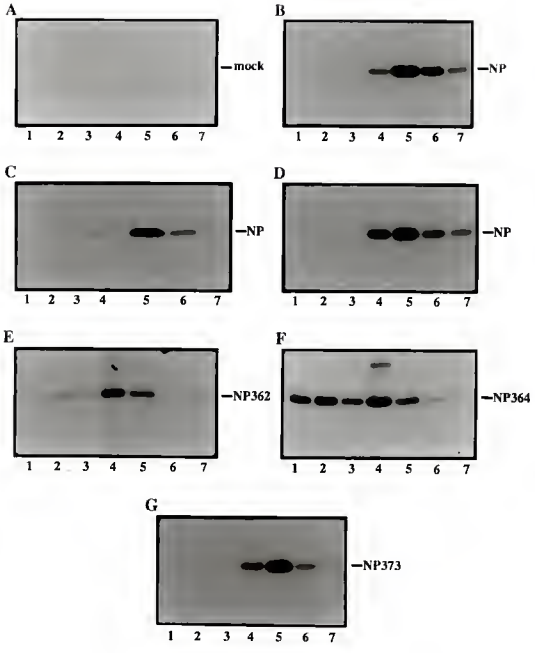
NP-NP Complex Formation Is Altered in Two Mutant NP Proteins

To measure self-assembly of the remaining mutant proteins NP362, NP364, and NP373, cell extracts containing the mutant proteins were analyzed on separate CsCl step gradients as described in Materials and Methods. Since the mutant NP364 had a limited period of stability (Fig. 11) and because the time required for self-assembly of the wt NP protein had not yet been established, pulse-chase experiments were performed. The wt NP protein was expressed in VVT7 infected A549 cells in triplicate and the cells were Tran^{35}S-labeled from 5.5 to 6 h p.t. in methionine- and cysteine-free

medium. Cell extracts were prepared immediately (pulse), following a 1.5 h chase, and after an 18 h chase in 10-fold excess methionine and cysteine medium. The proteins in the extracts were banded on separate CsCl step gradients, immunoprecipitated, and separated by SDS-PAGE as described in Materials and Methods. As shown in Figure 15B, the wt NP protein labeled in the pulse was found primarily in fraction 5 at the 30%-40% interface demonstrating the majority of the NP protein had self-assembled into nucleocapsidlike particles during the 30 min pulse. As expected, the self-assembled NP protein was stable for the 1.5 h and 18 h chase (Fig. 15C and 15D, respectively). It was shown that in untransfected cell extracts only a small amount of VVT7 proteins in the first four fractions reacted nonspecifically with the α-SV antibody (Fig. 15A). Since the wt NP protein self-assembled during the time that the mutant NP364 was shown to be stable, the mutant was tested for self-assembly.

The NP364 protein was expressed in A549 cells, radiolabeled for 30 min at 5.5 h p.t., cell extracts prepared following a 1.5 h chase, and the proteins banded on CsCl as described above. In contrast to wt NP, the mutant NP364 protein was found in similar amounts in fractions 1 to 5 (Fig. 15F). These results indicate that NP364 was not forming organized nucleocapsidlike particles, although some nonspecific NP-NP interaction did occur since the protein sedimented at variable rates. The higher molecular weight

Figure 15. CsCl step gradient centrifugation of the self-assembled wt and mutant nucleocapsidlike particles. A549 cells (4.8×10^6) were VVT7-infected and transfected with no plasmids (mock), the wt (NP), or mutant NP (2 μg) plasmid as indicated. At 5.5 h p.t. the cells were either pulse-labeled with Tran^{35}S-label for 30 min (B, C, D, and F) and cell extracts prepared immediately (B), following a 1.5 h chase (C and F), or a 18 h chase (D); or the cells were radiolabeled from 6-18 h p.t. (A, E, and G) before harvesting, as described in Materials and Methods. The cell extracts were analyzed separately by sedimentation on CsCl step gradients as described in Materials and Methods. Seven individual fractions were collected from the top of the gradient and the pellet was resuspended in the last fraction. Samples were analyzed by immunoprecipitation with an α-SV antibody as described in Materials and Methods. Sedimentation was from left to right and the positions of the wt and mutant NP proteins are indicated.


A
mock
1 2 3 4 5 6 7
B
NP
1 2 3 4 5 6 7
C
NP
1 2 3 4 5 6 7
D
NP
1 2 3 4 5 6 7
E
NP362
1 2 3 4 5 6 7
F
NP364
1 2 3 4 5 6 7
G
NP373
1 2 3 4 5 6 7

band in fraction 4 is a vaccinia virus protein (Fig. 15A) nonspecifically immunoprecipitated with the α-SV serum.

For NP362 and NP373 the cells were radiolabeled for 12 h in 10% methionine and cysteine medium cell extracts analyzed as described above. Self-assembled NP373 was found primarily in fraction 5, identical to self-assembled wt NP protein, demonstrating that the mutant NP373 protein self-assembled into nucleocapsidlike particles and is evidence that it is forming NP-NP interactions (Fig. 15G). Whereas, the mutant NP362 was found in similar amounts in both fractions 4 and 5, suggesting that perhaps self-assembly was occurring, but the protein-protein interactions are not stable, or never assembling correctly (Fig. 15E). A more precise separation of the protein structures is possible with the linear CsCl gradients, so the radiolabeled NP362 and wt NP proteins were banded on separate linear CsCl gradients, individual fractions collected, and analyzed as described in Materials and Methods.

In contrast to wt NP and NP370, NP362 banded at two positions in the gradient (Fig. 14D). One was at the top of the gradient (fractions 2-4) and the other at a density of 1.27 gm/cc (Fig. 14D). A parallel gradient containing self-assembled wt NP was shown to band at a density of 1.29 gm/cc (Fig. 14D). These results show that the mutant NP362 protein is not self-assembling into organized nucleocapsidlike particles as seen with wt NP.

Discussion

A summary of the properties of the wt and mutant NP proteins is shown in Figure 16. Minimally, the NP protein has been identified as an essential component of the NP-P and NP-NP complexes, as well as the NP-RNA complex, that are required for *in vitro* RNA replication (Horikami et al., 1992; Buchholz et al., 1993; Curran et al., 1993). Biochemical assays were used to measure the protein-protein interactions and an *in vitro* RNA replication assay was used to assess the NP-RNA interaction. Contrary to previous findings, our results show that the NP-P domain can be separated genetically from the NP-NP and NP-RNA domains, where the residues involved in the NP-NP and NP-RNA domains appear to be distinct.

The mutants NP362 and NP370 were both inactive in RNA replication *in vitro* (Fig. 16), yet their ability to form protein-protein complexes were different. The negative phenotype in replication with the mutant NP370 was not due to disruption of either the NP-P or NP-NP complex formation, which is evidence that the mutant protein structure was not globally disrupted. Additionally, we showed that the self-assembled NP370 nucleocapsidlike particles bound the P-L complex, suggesting the self-assembled particles are correctly formed. Based on this data, we propose that in the mutant NP370 the NP-RNA interaction was disrupted. Initially, the observed density (1.30 gm/cc) of self-assembled NP370 appears to contradict this hypothesis, unless

NP PROTEIN	COMPLEX FORMATION			VIRAL RNA REPLICATION (%) IN VITRO
	NP:P	NP:NP	P/L:NUC	α^{32}P Label
NP	+	+	+	100
NP362	+	+/-	ND	0
NP364	ND	-	ND	ND
NP370	+	+	+	0
NP373	+	+	ND	200

Figure 16. A summary of the wt and mutant NP protein-protein interactions and *in vitro* DI-H RNA replication data. The P/L:NUC results are from Fig. 8 and data not shown, and the remaining data are from Figs. 12-15.

you consider the type of RNA encapsidated. Self-assembled nucleocapsidlike particles that were expressed from plasmid DNA and banded at the density of NP-RNA molecules have been shown to contain a heterogeneous population of nonspecific RNAs (Buchholz et al., 1993). Whereas, initiation of encapsidation of specific SV genomic RNA, as shown previously for VSV (Blumberg et al., 1983; Moyer et al., 1991; Smallwood and Moyer, 1993), may require recognition of a specific signal by the NP protein. The highly conserved charged residues 370 and 371, which were substituted with alanine in NP370 could be required for this specific interaction.

The mutant NP362 protein formed NP-P complexes, but the NP-NP complex, as measured by the lack of self-assembly into oraganized nucleocapsidlike structures, was disrupted (Figs. 14D and 15E). The population of NP362 protein found in fractions 2-4 on the linear CsCl gradient most likely represents free protein that has not reached equilibrium (Fig. 14D). However, the portion of NP362 that banded at a density of 1.27 gm/cc suggests the mutant is forming some type of faster sedimenting protein-protein interactions. The lighter buoyant density suggests that this population does not contain RNA. One possibility is that this population represents unstructured NP362 aggregates, but this seems unlikely since you would expect a more heterogeneous distribution on the CsCl step gradients (Buchholz et al., 1993). A second more plausible possibility is that NP362 is still forming structured NP-NP oligomers, but that the NP-NP

interaction has been disrupted such that the domain for binding to RNA, even heterogeneous RNA, has been disrupted. However, these results do not allow us to distinguish if the faster sedimenting proteins are forming structured or unstructured oligomers. An RNA binding assay would permit us to assay for specific verses nonspecific NP-RNA interactions, without the need for the protein-protein interactions that are required for RNA replication. So far, this approach has not proved successful. Since the substrate for encapsidation in replication *in vitro* is the NP-P complex, it is possible that in the absence of P only nonspecific encapsidation occurs (Horikami et al., 1991; Buchholz et al., 1993).

The entire region from aa 362 to 371 (Phe, Leu, Leu, Gly, Gln, Ala, Val, Ala, Lys, and Asp) is conserved between the four parainfluenza viruses (Fig. 17; HPIV-1, SV, HPIV-3, and BPIV-3). The Phe at aa 362 and the Lys and Asp at aa 370 and 371, respectively, are conserved between 12 of 13 paramyxoviruses and the Gly at aa 365 is conserved in all 13 paramyxoviruses (Fig. 17). Although the NP protein, like the RNA-binding protein of *Escherichia coli* (*E. coli*) ColE1 plasmid-encoded Rop protein, does not show any structural or sequence homology to known RNA-binding domains (RBD) (Predki et al., 1995; Burd and Dreyfuss, 1994), this conserved sequence contains aa that have been shown to be required for protein-RNA interactions. Site-directed mutagenesis of several RNA binding proteins, including the *E. Coli* Rop and rho proteins and the U1 small nuclear ribonucleoprotein (U1

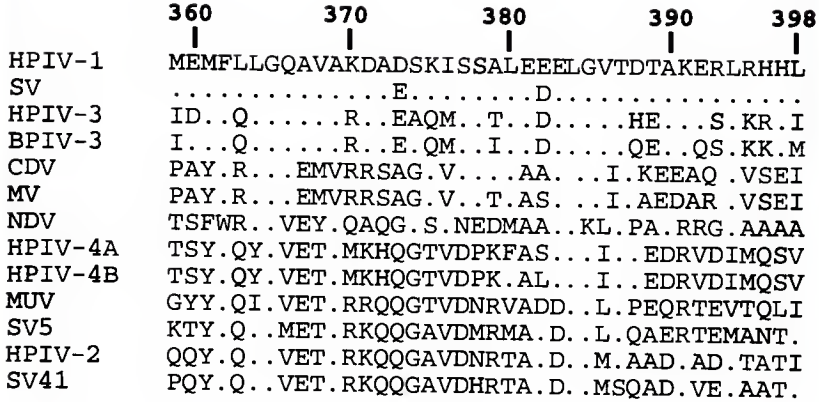


Figure 17. Amino acid alignment of selected NP sequences adapted from "Molecular evolution of paramyxoviruses: Nucleotide sequence analyses of the human parainfluenza type I virus NP and M protein genes and construction of phylogenetic trees for all the human paramyxoviruses." Arch. Virol. 124:255-268 (Miyahara et al., 1992). The aa identical to HPIV-1 NP are indicated by dots. The numbering at the top represents residues 359 to 398 relative to the SV NP aa sequence. The abbreviations represent the following viruses: Human parainfluenza type 1, 2, 3, 4A, and 4B (HPIV-1, HPIV-2, HPIV-3, HPIV-4A, and HPIV-4B, respectively), bovine parainfluenza type 3 (BPIV-3), simian virus 5 and 41 (SV5 and SV41, respectively), canine distemper virus (CDV), measles virus (MV), Newcastle disease virus (NDV), mumps virus (MUV).

snRNP), has identified both charged, aromatic, and hydrophobic residues as involved in RNA binding (Burd and Dreyfuss, 1994; Predki et al., 1995). For the *E. coli* Rop and rho proteins, specific phenylalanine residues have been shown to be essential for binding RNA (Brennan and Platt, 1991; Predki et al., 1995). Photocrosslinking studies on the type C1/C2 RBD has also identified a specific phenylalanine residue as being directly involved in RNA binding or forming the RNA binding domain, via protein-protein interaction, that binds to the RNA (Amrute et al., 1994).

It may be that the NP-NP and the NP-RNA domains overlap and that disruption of either domain could affect the other. The phenotype of the mutant NP362 is consistent with this model (Fig. 16), and also suggests that the NP-NP binding domain (aa 257-358), which was mapped using additional assays (Figs. 19 and 23) may be extended up to aa 369. These hypotheses will be discussed in greater detail in chapter 5. Self-assembly of NP370 into nucleocapsidlike particles (Fig. 14B and C), but its inability to support replication *in vitro* (Fig. 11B) shows that if the NP-NP and NP-RNA domains overlap, specific residues are involved in each interaction and that they can be separated genetically. The charged aa at 370 and 371 delineate the end of the NP-RNA region since substitution of the charged aa at positions 373 and 375 (NP373) did not disrupt protein-protein interactions or RNA replication (Fig. 16).

The inability of NP364 to self-assemble into organized nucleocapsidlike particles is likely due to improper folding since it is subject to proteolysis in the cell (Cunningham and Wells, 1989). The combination of instability and lack of self-assembly in the mutant NP364 suggests a correlation between these two, since four additional mutant NP proteins that have been shown to be unstable also do not self-assemble (Fig. 26, and data not shown). The heterogeneous distribution of NP364 protein in the CsCl gradient (Fig. 15F) has been described previously in NP deletion mutants (Buchholz et al., 1993). Examination of cell extracts expressing these NP deletion mutants (group I) by electron microscopy and immunogold labeling found only large unstructured aggregates. Interestingly, the NP deletion mutants examined were reported to be expressed as stable proteins. Based on these data, we propose that NP364 is forming nonspecific NP-NP interactions resulting in the formation of large aggregates.

In conclusion, site-directed mutagenesis has shown that although the NP-NP and NP-RNA domains may overlap, separate domains exist for these and the NP-P and Nuc-P/L domains. Development of additional experimental assays will need to be developed to determine if the NP protein recognizes a specific sequence or structure for initiation of encapsidation and whether the NP-NP and NP-RNA domains are separate or combined.

CHAPTER 5
IDENTIFICATION OF THE NP SELF-ASSEMBLY DOMAIN

Introduction

One of the first indications of an NP-NP interaction was that the virion RNA within the nucleocapsid was resistant to nuclease digestion (Heggeness et al., 1980). This suggested that as the NP protein encapsidated the nascent RNA it (the NP protein) was binding to the adjacent NP proteins, such that the RNA was completely protected. Also, it has been shown for VSV that once encapsidation has been initiated it is highly cooperative, suggesting an NP-NP interaction (Blumberg et al., 1983). The most compelling evidence for NP-NP interaction was shown by the self-assembly of the individually expressed SV NP protein into nucleocapsidlike particles (Buchholz et al., 1993). Analogous to the results obtained by Curran et al. (1993) with the NP deletion mutants in replication *in vitro*, Buchholz et al. (1993) found that the N-terminal 400 aa were also required for self-assembly into nucleocapsidlike particles. Because of the inherent difficulties associated with interpreting data collected using deletion mutants it was possible that individual domains existed within these first 400 aa and that the NP-NP domain could be isolated and identified.

The importance of characterizing the NP-NP binding domain is that the NP-NP interaction appears to be required for both structural and functional roles in SV RNA replication. Additionally, the ability of NP to encapsidate heterologous viral sequences during self-assembly represents an area that could be explored in further detail. Previous analyses have suggested that the NP protein has a complex tertiary structure, but limited evidence for specific conformations is known. By using different approaches to identify domains we hope to shed some light on the structure and function of the NP protein.

Three criteria were used to identify aa 258-357 of the NP protein as a region containing an NP-NP binding domain. First, it was necessary to identify the protein-protein complexes formed by the maltose-binding protein-NP1 (MBP.NP1) fusion protein. Second, we demonstrated that the fusion protein inhibits SV DI-H RNA replication *in vitro*. These two approaches identified the aa from 258-357 of the NP protein that were subsequently targeted for site-directed mutagenesis in the full-length NP protein. Third, we showed that point mutations within aa 258-357 specifically disrupted self-assembly and activity of NP protein.

Results

Construction of the MBP-NP1 Fusion Protein and Analysis by Sedimentation through Glycerol

Previous experiments have suggested that NP expressed alone, i.e., self-assembled, is found primarily in the pellet

fraction of a cell extract (Horikami et al., 1992) and this has been confirmed using linear glycerol gradient analysis of the NP protein (data not shown). Coexpression of the SV P and NP proteins leads to the formation of a soluble NP-P complex that can be identified by sedimentation analysis (Buchholz et al., 1994; Curran et al., 1995; Horikami et al., 1992). Based on this knowledge we predicted that if the domain responsible for NP-NP binding was fused to a soluble monomeric protein, the resulting association between the NP domains in the fusion protein would lead to the formation of protein-protein complexes. Using sedimentation analysis of the fusion protein we could identify the region of the NP protein involved in the NP-NP interaction.

The soluble maltose binding protein (MBP) was chosen as the fusion protein substrate. Fusion proteins with MBP have been used previously to identify RNA-binding domains in eukaryotic proteins (Anderson et al., 1993). To determine which regions of the NP protein would be isolated and fused to the MBP protein we took advantage of an amino acid alignment that identified a central conserved region (CCR) in 13 different paramyxovirus NP proteins (Fig. 18A) (Miyahara et al., 1992). In the SV NP protein this region encompasses aa 258 to 357 (underlined in Fig. 18A) and the first MBP-NP fusion protein (MBP.NP1) was created using this region (Table 3; Fig. 18B, region 1; and Fig. 20A). As a means for creating this and the subsequent fusion proteins, the *E. coli MalE* gene that codes for MBP was cloned downstream of the T7

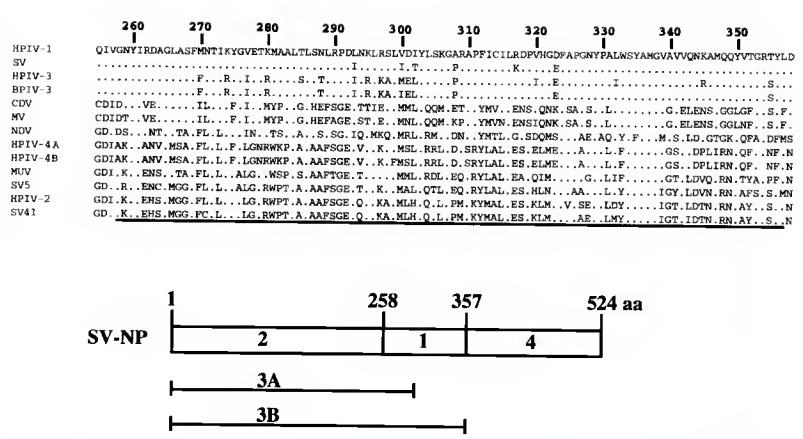


Figure 18. Amino acid alignment of selected NP sequences and a schematic representation of the NP protein A) Amino acid alignment adapted from "Molecular evolution of paramyxoviruses: Nucleotide sequence analyses of the human parainfluenza type I virus NP and M protein genes and construction of phylogenetic trees for all the human paramyxoviruses." Arch. Virol. 124:255-268 (Miyahara et al., 1992) as described in Fig. 17. Residues 255 to 358 relative to the SV aa NP sequence are shown and the central conserved region (CCR, aa 258-357) is indicated by underlining. B) Sections of the NP protein (1, 2, 3A, 3B, and 4) that were cloned downstream of the MBP in the construction of MBP.NP fusion proteins. The numbers above the drawing represent the relative position of NP amino acids. Section 2 contains the N-terminal 2-255 aa, and section 1 is the CCR (aa 258-357), as defined in (A). Section 3A contains aa 2-294, section 3B combines region 1 and 2 (aa 2-357), and section 4 contains the C-terminal 358-524 aa.

promoter in a modified pGEM3 vector (pMBP) as described in Materials and Methods and Table 3. The pMBP plasmid was used as the parent vector for cloning downstream of MBP various regions of NP (1, 2, 3A, 3B, and 4; Fig. 18B) encompassing the entire protein as described in Table 3.

To assay for the NP-NP interaction with the MBP.NP1 fusion protein (NP aa 258-357), we used sedimentation analysis on linear glycerol gradients. As controls and molecular weight markers we also sedimented the wt NP protein, MBP alone, and SV DI-H nucleocapsids. The wt NP, MBP.NP1, and MBP proteins were expressed individually in VVT7-infected and transfected A549 cells in the presence of Tran^{35}S-label as described in Materials and Methods. Radiolabeled cell extracts were prepared at 18 h p.t. in SV RM salts with 0.25% NP40 as described in Materials and Methods. The ^{35}S-labeled cell extracts and a ^{32}P-labeled nuclease resistant DI-H nucleocapsid product from an *in vitro* replication reaction were separately analyzed on linear glycerol gradients as described in Materials and Methods (Sprague et al., 1983).

The relatively brief centrifugation time allowed us to fractionate fast from slowly sedimenting structures to visualize protein-protein interactions. Following centrifugation, individual fractions were collected from the top of the gradient, ^{35}S-labeled proteins were immunoprecipitated with an α-SV antibody (wt NP), or an α-MBP antibody (MBP and MBP.NP1) and analyzed by SDS-PAGE. The

amount of the wt NP, MBP, or MBP.NP1 proteins in each fraction was quantitated on the phosphorimager as described in Materials and Methods and plotted as shown in Fig. 19A and B. For the gradient containing the ^{32}P-labeled DI-H nucleocapsids, the TCA-precipitable radioactivity in each fraction was determined and is shown in Figure 19A.

The SV DI-H nucleocapsids sedimented primarily in fractions 13 and 14 as expected (Sprague et al., 1983) (Fig. 19A). It has been shown previously that SV DI-H nucleocapsids sediment at a value of 14S (Carlsen et al., 1985; Baker and Moyer, 1988). The majority (53%) of the wt NP protein sedimented in a broad peak (fractions 17-22) with about 7% overlapping the position of DI-H nucleocapsids (fractions 13 to 16). A population of NP complexes was also found in the pellet (15%). The monomeric NP protein is expected to have a sedimentation value of approximately 4S (Howard and Wertz, 1989). This type of heterodisperse sedimentation pattern has been demonstrated previously for the assembled VSV N protein under the same sedimentation conditions (Sprague et al., 1983). Since the SV DI-H RNA is encapsidated by 235 molecules of the NP protein (Calain and Roux, 1992), it is clear that the NP protein expressed alone forms very large multimers. By EM analysis the majority of these multimers are organized, self-assembled nucleocapsidlike particles of varying lengths (Buchholz et al., 1993). Consistent with this is the data from isopycnic centrifugation on CsCl gradients which showed that the

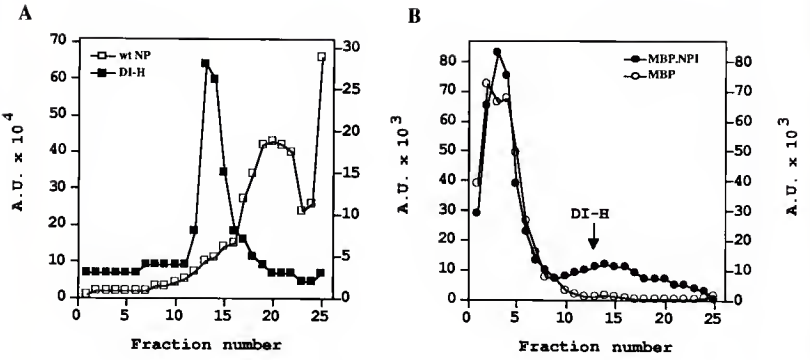


Figure 19. Glycerol gradient analysis of the wt NP protein, the MBP protein, the MBP.NP1 fusion protein, and a DI-H nucleocapsid. A549 cells (4.8 x 10^6) were VVT7-infected and transfected with the wt NP, MBP, or MBP.NP1 (2μg) plasmid and labeled with Tran^{35}S-label from 6-20 h p.t. as described in Materials and Methods. Cell extracts were prepared and analyzed separately by sedimentation on glycerol gradients as described in Materials and Methods. Sedimentation is from left to right. Samples were immunoprecipitated with either an α-SV (wt NP), or an α-MBP (MBP and MBP.NP1) antibody and analyzed by 9% SDS-PAGE. The amount of labeled NP protein (A) (□), MBP protein (B) (○), or MBP.NP1 protein (C) (●) in each fraction was quantitated on the phosphorimager and is depicted graphically. As a marker, the ^{32}P-labeled nucleocapsid products from an *in vitro* DI-H replication reaction were sedimented on a parallel gradient as described in Materials and Methods. The TCA-precipitable activity in each fraction was determined (■) and is plotted in (A).

majority of the expressed NP protein banded at the buoyant density of authentic nucleocapsids (1.30 gm/cc; Fig. 14C).

The MBP protein sedimented in fractions 1 to 6 (88%; Fig. 19B) with the majority in fractions 2 to 4 (56%; Fig. 19B) as expected for a soluble monomeric protein. It is noted that in the pMBP construct the first stop codon is in the vector sequence following the end of the MBP coding region and the multiple cloning sites for the construction of fusion proteins. Therefore, *in vivo* expression results in a MBP protein containing 414 aa, of which the first 352 aa actually code for the MBP. The sedimentation pattern of the fusion protein MBP.NP1 shows two peaks (Fig. 19B). First, a soluble protein population is found at the top of the gradient, but another faster sedimenting broad peak is found in the middle of the gradient from fractions 11 to 20. The data suggest that the NP CCR in the MBP.NP1 fusion proteins are interacting with each other. Based on the position of the second peak we suggest that the fusion protein is oligomerized and that the complexes range from similar to and larger than the size of the DI-H nucleocapsids containing 235 NP proteins. Approximately, 20% of the total MBP.NP1 protein is found in fractions 11 to 24, as compared to 2.6% of the control MBP in these same fractions, showing that the interaction is specific for NP1. The fact that the MBP.NP1 fusion protein is not found in the pellet fraction (Fig. 19B, fraction 25) under these sedimentation conditions suggests

that this interaction is not due to nonspecific aggregation of the fusion protein.

Since the data suggest that aa 258 to 357 of the NP protein contain an NP-NP interaction site(s), we wanted to define the boundaries of this site. Since this region is conserved within the paramyxoviruses, we first created a MBP fusion protein with the analogous region (aa 258 to 357) of the MV N protein (MBP.N1; Fig. 20A). Following restriction endonuclease digestion of the respective plasmids, *in vitro* transcription and translation produced full-length or truncated radiolabeled MBP.NP1 and MBP.N1 proteins (Fig. 20A) as described in Materials and Methods.

Instead of sedimentation on linear glycerol gradients a more facile approach was used for assaying protein-protein interaction. This was done by sedimenting 32% of the rabbit reticulocyte lysate containing the full-length or truncated proteins separately through 30% (v/v) glycerol and collecting the pelleted proteins in 2X lysis buffer as described in Materials and Methods. The pellets (Fig. 20C) and 4% of the total protein (Fig. 20B) in each rabbit reticulocyte lysate were analyzed by SDS-PAGE. In each case, proteins of the appropriate sizes were synthesized (Fig. 20B, top band in each lane). The faster migrating proteins are apparently early termination products. The amounts of the total and pelleted proteins were quantitated on the phosphorimager. Using this assay, the amount of MBP.NP1 protein that pelleted (Fig. 20C, lane 4) was 19 ± 3.6% (± S.E., n = 4) of the total

protein (Fig. 20B, lane 4). Similar results were obtained with the MBP.N1 protein (Fig. 20B and C, lanes 2).

This value is very near the 20% of the MBP.NP1 protein found in fractions 11 to 24 on the linear glycerol gradient (Fig. 19B), thus the fraction that pellets can be used as a measure of protein-protein interaction. As a negative control, the soluble MBP (399 aa) did not pellet (0.31 ± 0.31% [± S.E., n = 7]) in this assay as expected (Fig. 20B and C, lanes 1). Therefore, for comparisons we have arbitrarily set the amount of the MBP.NP1 (and MBP.N1) protein that pellets through 30% glycerol at 100%. The percent protein in the pellet compared to its total for all of the other fusion proteins was normalized to this value and was used as a measure of the NP-NP interaction (Fig. 20A, Pellet(%)).

Removal of 33 or 63 aa from the C-terminus of NP1 (MBP.NP1-B or MBP.NP1-H) drastically reduced (15% and 5%, respectively) the amount of the fusion protein that pelleted through glycerol (Fig. 20A, B, and C; lanes 6 and 5, respectively). Likewise, only 21% of the truncated MBP.N1 fusion protein (MBP.N1-P) pelleted (Fig. 20A, B, and C; lanes 3). These results show that the CCR mediates the Sendai NP-NP and the measles N-N interaction in these proteins and removal of just one-third (NP) or one-half (N) of the aa from the C-terminus of this region significantly disrupts the interaction. To determine whether the aa from 257 to 295 of the NP protein are required for the NP-NP interaction, a

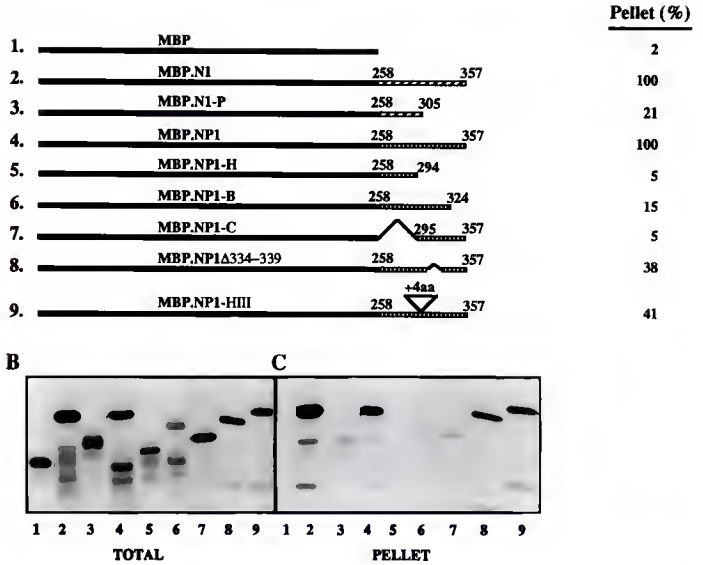


Figure 20. Sedimentation analysis of the MBP fusion proteins containing region 1 (CCR) of both the MV N protein (MBP.N1) and the SV NP protein (MBP.NP1) and their derivatives. A) Schematic representation of the MBP (▬), MBP.N1 (▬), and MBP.NP1 (▬) fusion proteins produced by *in vitro* transcription and translation in the presence of ^{35}S-methionine as described in Materials and Methods. The numbers above the protein refer to the first and last aa of the viral peptide in the fusion protein. The amount of the parent MBP.N1 or MBP.NP1 fusion protein that pelleted (compared to its total) through glycerol was set at 100%, and the percent of protein in the pellet compared to its total for all the other fusion proteins was normalized to this value. A) A sample of the radiolabeled protein was analyzed directly by 9% SDS-PAGE, or (B) a sample was pelleted through glycerol and the pellets were analyzed by 9% SDS-PAGE as described in Materials and Methods. The amount of radiolabeled protein was quantitated on the phosphorimager. The sample numbers in (B and C) correspond to those in (A).

fusion protein was constructed that had deleted these aa from the conserved region (MBP.NP1-C; Fig. 20A). The inhibition in the amount of MBP.NP1-C that pelleted (5%; Fig. 20B and C; lanes 7) suggested that the N-terminus of the CCR is also required for the NP-NP interaction.

Previously in our laboratory, two mutants in the central conserved region of the NP protein (NPHIII and NPΔ334-339) had been created, but neither mutant was expressed *in vivo* as a stable protein (data not shown). The mutant NPHIII contains an insertion of four aa at the HindIII restriction site (aa 296) and six aa were deleted in the mutant NPΔ334-339 from aa 334 and 339. To determine if these mutations affect binding, we fused the central conserved region (aa 258-357) of each of these mutants to the MBP (Fig. 20A). Each fusion protein (MBP.NP1HIII and MBP.NP1Δ334-339) was expressed *in vitro* and pelleted through glycerol as described above. The level of NP-NP interaction in each fusion protein was reduced by more than half relative to MBP.NP1 (38% and 41%, respectively; Fig. 20B and C, lanes 8 and 9). These results show that even small perturbations within the CCR of the NP protein disrupt the NP-NP interaction and suggest that a correlation may exist between protein stability *in vivo* and NP-NP interaction.

Analysis of the MBP.NP2, MBP.NP3A, MBP.NP3B, and MBP.NP4 Fusion Proteins by Sedimentation through Glycerol

The above data has identified aa 258-357 as containing an NP-NP binding site, and we wanted to test if other regions

of the NP protein had this capability. Regions encompassing the rest of the NP protein (Fig. 18, regions 2 through 4) were fused to the MBP, as described in Table 3, to assay for any NP-NP interaction. This was done for two reasons: one, there may be two sites for NP-NP interaction on the NP protein. One site may bind to the leading NP molecule and another may bind to the lagging NP molecule, permitting cooperative encapsidation of the viral RNA as well as self-assembly of nucleocapsidlike particles. Two, it is possible, but not probable, that any region of the NP protein when fused to the MBP can interact with itself.

Full-length (aa 2-255) and truncated derivatives of the MBP.NP2 protein were made by linearizing the plasmid downstream of or within the fusion protein coding region using unique restriction sites followed by *in vitro* transcription and translation of the protein (Fig. 21A and B). Sedimentation analysis showed that some degree of NP-NP interaction occurred with each of these fusion proteins, but that it was less than that with the MBP.NP1 fusion protein. Compared with MBP.NP1 as 100%, the fusions containing the first 255 aa, 187 aa, 130 aa, and 115 aa of NP2 gave 40%, 71%, 51%, and 62% binding, respectively (Fig. 21B and C, lanes 1-4). Again, the MBP alone did not pellet (2%; Fig. 21B and C, lanes 5) as expected. These results suggested that while the N-terminus of the NP protein also contains an NP-NP interaction site (minimally, aa 1-115), it may contain elements negatively affecting the interaction as well.

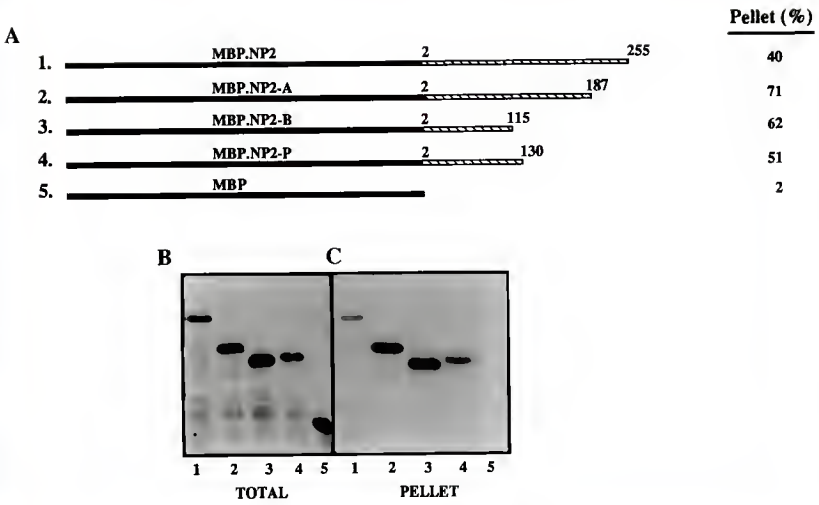


Figure 21. Sedimentation analysis of the *in vitro* synthesized MBP fusion protein containing region 2 (N-terminus) of the NP protein (MBP.NP2) and truncations of the MBP.NP2 fusion protein. A) Schematic representation of the MBP (▬), MBP.NP2 (▬▭), and truncated MBP.NP2 (▬▭) fusion proteins produced by *in vitro* transcription and translation in the presence of ^{35}S-methionine as described in Materials and Methods. The products were analyzed as described in Fig. 20 and the percent of protein in the pellet was normalized to MBP.NP1 as described in Fig. 20.

If the N-terminus of the NP protein does contribute to the NP-NP interaction one would expect that the combination of the N-terminus and the CCR would pellet through glycerol at levels equal to or even greater than with the CCR alone. To answer this question and to determine the effect of the C-terminal aa of the NP protein, the following fusion proteins were also analyzed: MBP.NP3B (NP aa 2-357), MBP.NP3A (NP aa 2-294), and MBP.NP4 (NP aa 358-524) (Fig. 22A). These proteins were expressed and assayed for NP-NP interaction as described above and the amount of protein that pelleted was compared to MBP.NP1 (set at 100%).

Interestingly, the combination of the N-terminus and CCR of the NP protein (MBP.NP3B) was less than the CCR alone (MBP.NP1), but still gave a significant level of interaction (80%; Fig. 22B and C, lanes 2). It appears as though there is some inhibition of the NP-NP interaction in this fusion similar to what was observed with the MBP.NP2 protein. The MBP.NP3A fusion protein was severely inhibited in its ability to form an NP-NP interaction (10%; Fig. 22B and C, lanes 1), demonstrating that truncation of the CCR abolished interaction of the entire fusion protein, including that of the NP2 region. Subsequently, linear glycerol gradient analysis of MBP-NP2 showed that the contribution of aa 2-255 to the formation of protein-protein complexes was at most secondary. The C-terminal 167 aa of the NP protein do not contain an NP-NP binding site as shown by the lack of protein in the pellet (1%, Fig. 22B and C, lanes 3).

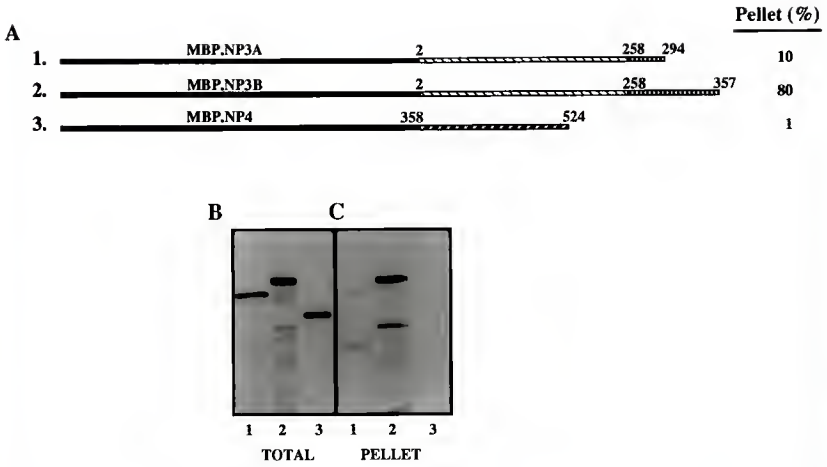


Figure 22. Sedimentation analysis of the *in vitro* synthesized MBP fusion proteins containing regions 3A, 3B, and 4 of the NP protein (MBP.NP3A, MBP.NP3B, and MBP.NP4, respectively). A) Schematic representation of the MBP.NP3A and MBP.NP3B (▬), and the MBP.NP4 (▬) fusion proteins produced by *in vitro* transcription and translation in the presence of ^{35}S-methionine as described in Materials and Methods. The products were analyzed and the percent of protein in the pellet was normalized to MBP.NP1 as described in Fig. 20.

A summary of the above data on the interaction of the MBP-NP fusion proteins is shown in Figure 23. The CCR (NP1, aa 258-357) of the NP protein has been identified as a probable site for NP-NP interaction. The data show that any large deletion within this region severely inhibits and small insertions or deletions reduced the interaction by more than 50%. Furthermore, the N-terminal aa 2-115 may also contain an NP-NP interaction site; however, the C-terminus (aa 358-524) of the NP protein does not.

Inhibition of SV-DI-H RNA Replication by the MBP-NP1 Fusion Protein

Since aa 258-357 (CCR) appeared to contain an NP-NP binding domain, we next wanted to test if this interaction had biological significance, perhaps to inhibit DI-H replication *in vitro*. VVT7-infected A549 cells were transfected with no plasmids or the NP, P, and L plasmids and increasing amounts of either the pMBP.NP1 or the pMBP plasmid as indicated at the top of Figure 24A. *In vitro* replication was assayed as described previously by incubating the cell extracts with dd SV DI-H and [α-^{32}P]CTP. The radiolabeled, nuclease resistant replication products were banded on CsCl gradients, the product RNA extracted and analyzed by gel electrophoresis (Fig. 24A). Protein expression was monitored by immunoblot analysis on 10% of the cell extract with an α-SV and α-MBP antibodies as described in Materials and Methods (Fig. 24B).

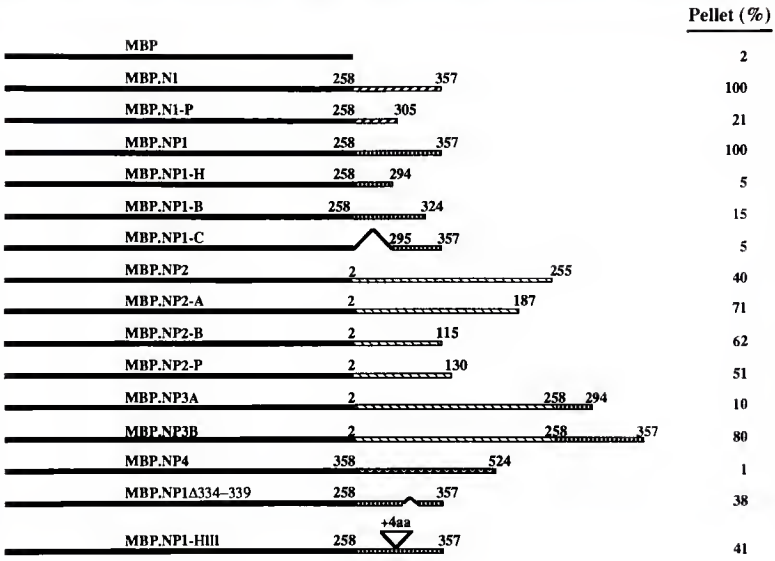


Figure 23. A summary of the sedimentation data of the MBP fusion proteins. Schematic of the fusion proteins and percent NP-NP interaction as indicated by the percent of protein pelleted as described in Figs. 20, 21, and 22.

DI-H replication was supported by the NP, P, and L proteins (100%, Fig. 24A and B, lane 2) and was dependent on the expression of these proteins (Fig. 24A and B, lane 1). Addition of increasing amounts of the pMBP plasmid that corresponded with increasing MBP expression (Fig. 24B, lanes 6, 7, and 8, respectively) had little or no effect on DI-H replication (85%, 114%, 86%; Fig. 24A, lanes 6, 7, and 8). The presence of increasing MBP.NP1 protein (2 µg and 3 µg of plasmid transfected; Fig. 24B, lanes 4 and 5, respectively); however, decreased DI-H replication to 50% and 22%, respectively (Figs. 24A and 24B, lanes 4 and 5). These data show that just the CCR (aa 258-357), as part of a fusion protein, can inhibit SV-DI-H replication *in vitro*, and this inhibition is specific for the viral sequences since MBP alone does not inhibit. We propose that the MBP.NP1 fusion protein is interfering specifically with the native NP-NP interaction during replication, because we have also shown that the MBP-NP1 fusion does not bind to the P protein or to synthetic RNA (data not shown).

Point Mutations within the CCR Disrupt Self-Assembly of Nucleocapsidlike Particles

As described previously, the NP-NP interaction can be measured by the self-assembly of the NP molecules into nucleocapsidlike particles. Since we have identified the CCR of the NP protein as an NP-NP binding domain in a fusion protein, we wanted to confirm this in the native NP protein. This was done by constructing point mutations within the CCR

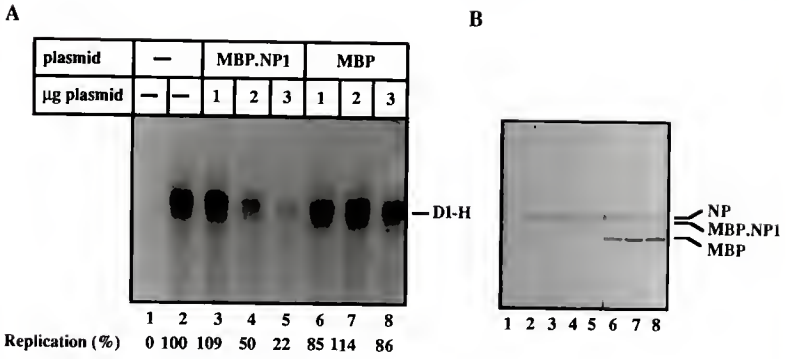


Figure 24. Inhibition of *in vitro* DI-H RNA replication by the MBP.NP1 fusion protein. B) A549 cells were infected with VVT7 and transfected with no plasmids (-), or cotransfected with the P and L plasmids together with the wt NP plasmid, as described in Fig. 2. As indicated at the top, transfection mixes also contained increasing amounts of either the MBP.NP1 or MBP plasmid. Cytoplasmic cell extracts were prepared at 18 h p.t. and *in vitro* replication was assayed as described in Fig. 2. The position of the DI-H RNA is indicated and the amount of product DI-H RNA was quantitated on a phosphorimager and reported as a percentage of replication with the NP, P, and L proteins (lane 2, 100%). C) Immunoblot analysis on samples (10%) of the cytoplasmic cell extracts used for *in vitro* replication with both the α-SV and α-MBP antibodies as described in Materials and Methods. The positions of the NP, MBP.NP1, and MBP proteins are indicated. The sample numbers in (B) correspond to those in (A), respectively.

of the wt NP gene (Fig. 25), at conserved hydrophobic residues, by random-primed PCR mutagenesis as described in Materials and Methods and Table 2. The mutant proteins were then analyzed for self-assembly and biological activity.

The wt and mutant NP proteins were expressed in VVT7-infected and transfected A549 cells and protein stability was determined using pulse-chase analysis as described in Materials and Methods. The NP mutants, NP324-1 and NP324-5, were stable only for approximately 1.5 h following the pulse (data not shown). These mutants contained a Phe to Val or Phe to Ile substitution at aa 324, respectively. The NP mutants, NP260-1, NP299-5, and NP313-2 were each stable to an 18 h chase (data not shown). Based on these results, cell extracts used for further analysis of the mutant proteins were prepared during periods of stable protein expression.

Self-assembly of the mutant proteins was determined by expressing the proteins individually in VVT7-infected and transfected cells as described in Materials and Methods. The mutants NP324-1 and NP324-5 were radiolabeled for 30 min at 5.5 h p.t. and the cell extracts were prepared following a 1.5 h chase in 10-fold excess methionine and cysteine medium. Cell extracts containing the steady-state radiolabeled wt and remaining mutant NP proteins (NP260-1, NP299-5, and NP313-2) were prepared at 18 h p.t. The proteins were banded on separate CsCl step gradients, individual fractions were collected from the top of the gradient, immunoprecipitated with an α-SV antibody and analyzed by SDS-PAGE (Fig. 26A-F).

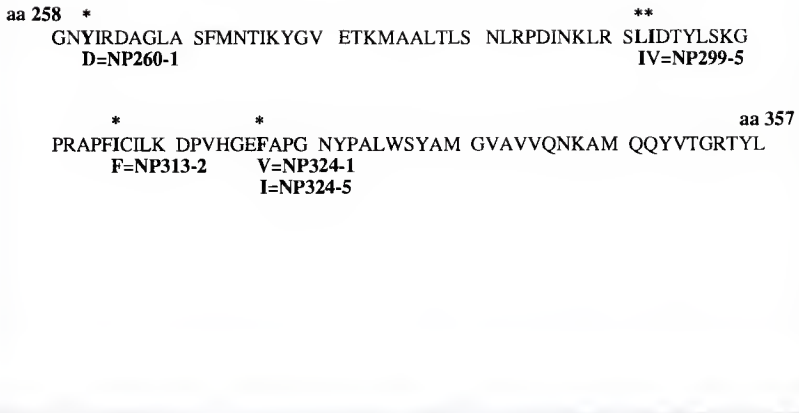


Figure 25. The amino acid sequence of the CCR (aa 258-357) of the NP protein and the random-primed site-directed NP mutants. The mutations are indicated by asterisks above the one-letter aa code and the mutant residues are shown below. Both the wt and the mutant aa are highlighted by bold lettering. The mutant is named according to the number of the mutated residue and the order in which it was isolated, i.e., in NP299-5, aa 299 was the first mutated aa and it was the fifth clone isolated at this position.

As the positive control, wt NP was shown to band at the position of nucleocapsids (fraction 5, 30-40% interface) (Fig. 26A).

The mutant proteins NP299-5 and NP313-2 formed nucleocapsidlike particles as shown by the majority of the protein banding in fraction 5 (Fig. 26C and D), demonstrating that the conservative substitutions at aa 299 and 313 did not disrupt self-assembly. This result for NP313-2 was confirmed by linear CsCl gradient analysis where the protein banded at a density of 1.30 gm/cc, identical to self-assembled wt NP (data not shown). The mutant NP324-1 and NP324-5 proteins were found primarily in the first two fractions of the gradient with a minor proportion in fractions 3 and 4 (Fig. 26E and F) and, thus, did not self-assemble into nucleocapsidlike particles. These results also suggest that self-assembly of NP may be coupled to protein stability under these conditions. The higher molecular weight bands are VVT7 proteins that were nonspecifically immunoprecipitated with the α-SV antibody in some experiments (see Fig. 15A and F).

Substitution of Tyr with Asp at aa 260 also disrupted NP-NP interaction, but to a lesser extent than the mutations at aa 324. The mutant NP260-1, like NP362 (Fig. 15E), was consistently found in both fractions 4 and 5, suggesting either that the NP-NP interaction is not forming correctly or that the complexes are forming, but are not stable and are dissociating. Mutants NP260-1, NP299-5, and NP313-2, are

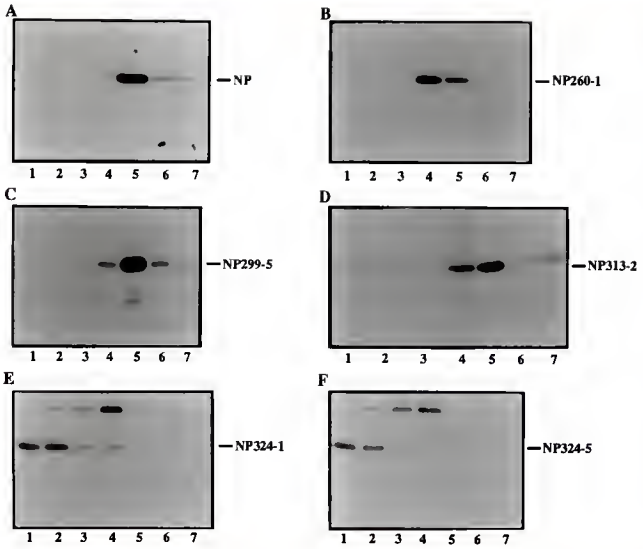


Figure 26. Analysis of the wt NP and mutant NP proteins by CsCl step gradient centrifugation. A549 cells (4.8×10^6) were VVT7-infected and transfected with the wt (NP) or the mutant NP (2 µg) plasmid, as indicated. The cells were either Tran^{35}S-labeled from 5.5-17.5 p.t. (NP, NP260-1, NP299-5, and NP313-2), or from 5.5-6.0 h p.t. (NP324-1 and NP324-5). Cell extracts were prepared at 17.5 h p.t. or following a 1.5 h chase, respectively, as described in Materials and Methods. The radiolabeled cell extracts were analyzed by sedimentation on separate CsCl step gradients, fractions were collected from the top, and the pellet was resuspended in the last fraction (sedimentation is from left to right). Samples were immunoprecipitated with an α-SV antibody and analyzed by 9% SDS-PAGE as described in Materials and Methods. The positions of the wt and mutant NP proteins are indicated.

apparently correctly folded proteins since they are stably expressed and all form NP-P complexes (data not shown).

Three of the CCR Mutants Are Inactive in DI-H RNA Replication *in Vitro*

Each of the stable mutant proteins NP260-1, NP299-5, and NP313-2 were also tested for biological activity. Cell extracts containing the expressed P and L proteins (viral polymerase) with the wt or mutant NP proteins were incubated with dd DI-H template in the presence of [α-^{32}P]CTP and the replication products analyzed by gel electrophoresis (Fig. 27). Each of the wt and mutant NP proteins and the P protein were expressed at significant levels as shown by immunoblotting (data not shown).

DI replication with the wt NP protein was significant (Fig. 27, lane 2) and dependent on the expression of the SV proteins (lane 1). Each of the mutant NP proteins, NP260-1, NP299-5, and NP313-2, was completely inactive in RNA replication (Fig. 27, lanes 5, 4, and 3, respectively). Since the mutants NP299-5 and NP313-2 can form NP-NP and NP-P complexes, these data suggest that the Leu and Ile at positions 299 and/or 300, and the Ile at position 313 are important for binding to the nascent RNA. For NP260-1 (Tyr to Asp), these results can not distinguish whether it is the disruption of the NP-NP interaction (Fig. 26B) or the disruption of RNA binding that is responsible for the defective RNA replication *in vitro*.

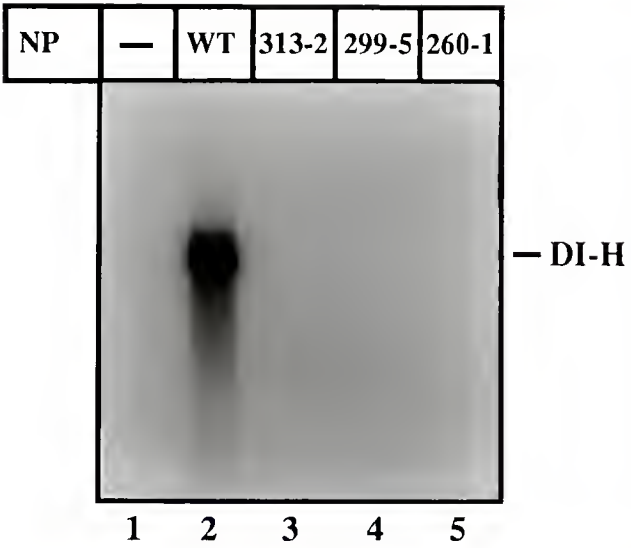


Figure 27. *In vitro* DI-H RNA synthesis with the CCR mutant NP proteins. A) A549 cells (4.8 x 10^6) were infected with VVT7 and transfected with no plasmids (-), or cotransfected with the P and L plasmids together with the wt or mutant NP plasmid, as indicated at the top, and described in Fig. 2. Cytoplasmic cell extracts were prepared at 18 h p.t. and *in vitro* replication was assayed as described in Fig. 2. The position of the DI-H RNA is indicated.

Discussion

We have used three diverse approaches to identify the NP-NP binding domain. First, MBP fusions with isolated regions of the NP protein identified aa 258-357 (CCR) as containing an NP-NP binding domain (Figs. 19 and 20). Additionally, the aa in the N-terminus appear to contribute to the formation of protein-protein interactions, but this contribution appears weaker. Significant amounts of truncated MBP-NP2 fusions containing aa 2-115 or 2-187 pelleted through glycerol (62% or 71%), but fusions containing aa 2-130 or 2-255 were reduced by approximately 50% or more compared to MBP-NP1. One explanation is that within the N-terminus of NP, elements are present that also inhibit the NP-NP interaction. The C-terminus of the NP protein did not yield an interaction (Fig. 22).

Clearly, the profile of the MBP-NP1 fusion protein (containing the CCR) under different sedimentation conditions identified protein-protein interactions that were dependent on the viral sequences (Figs. 19B, 20B and C). This conserved region in MV N was also required for N1 binding (Fig. 20B and C). About 80% of either the SV NP1 or MV N1 proteins sedimented as apparent monomers. It is possible that a fraction of these fusion proteins was either improperly folded and could not form the interaction or the binding occurred, but was relatively unstable. Inhibition of SV DI-H *in vitro* replication by the MBP-NP1 fusion, but not

MBP alone, was further proof that the CCR contained a biologically relevant domain (Fig. 24A).

As a second approach, introduction of certain point mutations, specifically at residues 260 and 324 in the CCR of the full-length NP protein, disrupted self-assembly and were therefore required for the NP-NP binding (Fig. 26B, E, and F). The presence of the mutant NP260-1 protein, as well as the previously analyzed NP362, in both fractions 4 and 5 suggests that the mutants are forming nucleocapsidlike particles, but that the interaction is not stable and dissociates, and this alone could be responsible for the negative results in replication (Fig 27, lane 3; Fig 12, lanes 3 and 4). For a summary of the properties of the CCR mutants and the alanine-scanning mutants see Fig. 28. The identical phenotypes of NP260-1 and NP362 suggest that the Tyr and Phe residues, respectively, are part of a single domain or possibly separate domains with identical functions. The combined data indicates that the downstream boundary of the NP-NP binding domain extends past the identified CCR of aa 258-357. We propose that it extends to at least aa 365 or 369, since Ala substitutions at aa 364 and 365, but not at 370 and 371 disrupted self-assembly (Figs. 14C, 15F, and 28). Examination of the aa sequence alignment (Fig. 17) shows that aa 358-369 are conserved among the parainfluenza viruses; therefore, the CCR could be lengthened to include these additional residues.

	COMPLEX FORMATION			VIRAL RNA REPLICATION (%) IN VITRO
NP PROTEIN	NP:P	NP:NP	P/L:NUC	α^{32}P Label
NP	+	+	+	100
NP260-1	+	+/-	ND	0
NP299-5	+	+	ND	0
NP313-2	+	+	ND	0
NP324-1	ND	-	ND	ND
NP324-5	ND	-	ND	ND
NP362	+	+/-	ND	0
NP364	ND	-	ND	ND
NP370	+	+	+	0
NP373	+	+	ND	200

Figure 28. A combined summary of the wt and mutant NP protein-protein interactions and *in vitro* DI-H RNA replication data from the CCR NP mutants and Fig. 16. The data for the CCR mutants, NP260-1, NP299-5, NP313-2, NP324-1, and NP324-5 are from Figs. 26, 27, and the text. The data for the mutants, NP362, NP364, NP370, and NP373, are as described in Fig. 16.

Interestingly, the phenotypes of the mutants NP299-5 and NP313-2 are identical to the phenotype of the mutant NP370 in that each mutant forms NP-NP and NP-P complexes (Figs. 26C and D; 14C and 13, respectively), but each is inactive in DI-H replication *in vitro* (Fig. 27, lanes 3 and 4; Fig. 12, lanes 5 and 6). These results show that the aa at positions 299 and 313, which appear to be required for the SV NP-RNA interaction, are located within the NP-NP binding domain, yet the two functions can be genetically separated. As discussed with NP370, these mutants retain the ability to nonspecifically bind to heterogeneous RNA; therefore, we propose that these mutants are defective in the specific binding required for SV DI-H RNA replication. In the secondary or tertiary structure of the NP protein we propose aa 299, 313, and 370 may be juxtaposed forming one functional domain. Alternatively, these aa could be part of separate domains, that have the same function.

Mutation of Phe to Val or Ile at aa 324 totally disrupted self-assembly in the mutants NP324-1 and NP324-5, respectively, suggesting that the Phe at this position is very important for the NP-NP interaction (Fig. 26E and F). This result was somewhat surprising since the substitutions were conserved except for size. Substitution of the Phe with the bulky Leu residue would help determine the importance of size at this position. Their short half-life suggests that these mutant proteins could also be misfolded. Another hypothesis is that in the absence of either the NP-NP or NP-P

interaction, the NP protein is not stable. The dependence on protein-protein interaction for stability has been shown for the SV L protein, which is not stable unless it is coexpressed with the SV P protein (Smallwood et al., 1994).

From the combination of these results with other limited information available on the NP protein we propose the following model: the CCR is divided into two sides or faces by virtue of a hinge region that was identified by trypsin digestion of nucleocapsids into NP fragments, which remained associated with the nucleocapsid (Fig. 29) (Heggeness et al., 1981). The possible cleavage sites are found at either aa 295, 299, 306, 309, or 317, although the precise site was not identified (Morgan et al., 1984). Each face of the CCR contains NP-NP binding sites required for binding to the adjacent NP proteins. Another alternative is that the hinge allows each side of the CCR to come together and form one NP-NP binding domain. Similarly, the mutants identified in the specific binding of SV RNA (NP299-5 and NP313-2) would be positioned on one or two sides. In either case, this model would account for the two groups of mutants that have identical phenotypes (NP260-1 and NP362; or NP299-5 and NP313-2).

In conclusion, we have mapped the NP-NP binding domain to aa 258-369 and showed that residues apparently required for the specific binding of SV RNA are found both within and downstream of the NP-NP binding domain. The aa required for the NP-NP and NP-RNA interactions are distinct, since certain

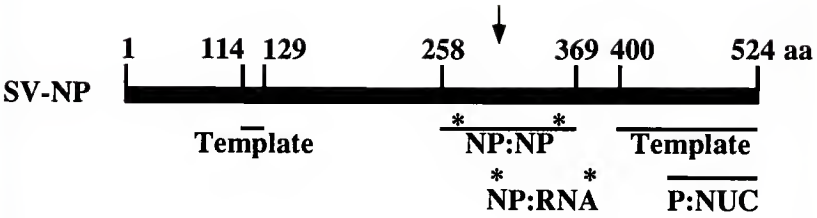


Figure 29. A schematic of the domains identified in the NP protein. The numbers above the protein (thick bar) represent the aa number of the NP protein. The thin bars indicate the location of the domain and the asterisks indicate the relative position of the individual aa that were shown to be specifically required for the interaction.

mutants can still bind NP, but not RNA (Figs. 28 and 29). Future experiments will be aimed at the further characterization of the structure of the NP-NP domain.

CHAPTER 6
CONCLUDING REMARKS

These studies have confirmed that the NP protein has a highly ordered tertiary structure, and they have also shown that individual protein domains can be identified. One of the striking characteristics is the presence of several nonlinear regions that appear to map on each side of a hinge region (Heggeness et al., 1981), suggesting that the separate residues could be in close contact in the folded protein (Fig. 29). The hydrophobic N-terminal 400 aa contain the NP-NP binding domain (aa 258-369), a template function domain (aa 114-129), as well as residues required for a putative NP-RNA binding site (aa 299, 300, 313, 370, and 371). While the first 400 aa are sufficient for the NP-NP and NP-RNA binding domains (Fig. 9), template function requires a C-terminal domain (aa 400-524) as well (Buchholz et al., 1993; Curran et al., 1993).

Based on the above data, which suggests two nonlinear regions form one domain, the identification of a P-Nuc binding site in aa 439-524 (Buchholz et al., 1994), suggests that an unidentified P-Nuc binding site may be present in the N-terminus of the NP protein. The highly charged character

of aa 439-524 suggests the P-Nuc interaction is hydrophilic; therefore, a possible P-Nuc binding site in the N-terminus is the charged region from aa 62-70. The specific function of the aa from 114-129 and 400-439 remains elusive, since although they are required for template function they are not required for the P-Nuc interaction (Fig. 9). A different approach will have to be designed to identify the function of this domain. One possibility is that this region is interacting with another viral protein or possibly a cellular protein. The yeast two-hybrid system could be used to test this hypothesis. We have shown that the NP protein interacts with another NP protein and the P protein in this system (Horikami et al., submitted).

The fact that the mutants NP299-5, NP313-2, and NP370 self-assemble into nucleocapsidlike particles, yet have a negative phenotype in RNA replication *in vitro*, suggested that the NP-NP binding domain and NP-RNA domain are separate. What is still not clear is whether the NP-RNA domain is dependent upon a functional NP-NP domain, since the mutants that were disrupted in the NP-NP interaction (NP260-1 and NP362), were of necessity negative in replication (Fig. 28). The lack of an *in vitro* RNA binding assay has limited the investigation of the NP-RNA interaction and may be an option that could be explored in the future. In my experience, the *in vitro* expression of the NP protein was less than optimal, with the majority of the protein not being full-length (data

not shown). A gel shift assay or photocrosslinking could also be used as approaches to identify NP-RNA interactions.

The exact location of the soluble NP-P binding site has not been identified in these or earlier studies, but it appears to be within the first 400 aa of the protein (Buchholz et al., 1991; Curran et al., 1993). None of the MBP.NP fusions tested, MBP.NP1, MBP.NP2, MBP.NP3A, and MBP.NP3B, bound to the GST-P protein (data not shown), suggesting that these NP fragments did not contain the complete NP-P domain. Our results would suggest that this domain is also likely to be nonlinear. In the absence of a crystal structure for the NP protein we must rely on the data obtained from these types of studies to shed light on the structure and function of the NP protein. Clearly, a combination of different types of approaches should be used to confirm the results of any one experiment.

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BIOGRAPHICAL SKETCH

The author was born in Long Beach, California. She received her elementary and secondary education in a small central Florida town and graduated from Citrus High School in 1979. She graduated from Central Florida Community College with an AS degree in nursing and received her professional registered nurse license from the State of Florida in 1981. She worked as a registered nurse at Munroe Regional Medical Center from 1981 to 1986. She received her BS from the University of Florida in 1990 and her major was microbiology and cell science with a minor in chemistry. She began her graduate studies at the University of Florida in 1990 and joined Dr. Sue Moyer's laboratory in 1991. She will graduate in the spring of 1996 and will pursue an academic postdoctoral position.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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